# LONGITUDINAL STUDY OF CYTOKINE STORM IN DENGUE PATIENT BY FLOW CYTOMETRY

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# Recommendation

This is to certify that the research work entitled "**LONGITUDINAL STUDY OF CYTOKINE STORM IN DENGUE PATIENT BY FLOW CYTOMETRY"** has been carried out by **Ms. Muskan Sainju** under my supervision.

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# Certificate of Evaluation

This is to certify that the thesis entitled "**LONGITUDINAL STUDY OF CYTOKINE STORM IN DENGUE PATIENT BY FLOW CYTOMETRY"** presented to evaluation committed by **Ms. Muskan Sainju** is found satisfactory for the partial fulfillment of Master of Science and Technology.


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# LIST OF ABBREVIATIONS

| | |
|---|---|
| CDBT | Central Department of Biotechnology |
| CDC | Central for Disease Control |
| CRF | Case Report Form |
| DENV | Dengue Virus |
| DF | Dengue Fever |
| DHF | Dengue Hemorrhagic Fever |
| DSS | Dengue Shock Syndrome |
| DVI | Dengue Virus Infection |
| ECDC | European Centre for Disease Prevention and Control |
| EDCD | Epidemiology and Disease Control Division |
| ELISA | Enzyme-linked Immunosorbent Assay |
| IL-1β | Interleukin- IL-1β |
| IL-6 | Interleukin-6 |
| IL-8 | Interleukin-8 |
| IL-10 | Interleukin-10 |
| IL-12p70 | Interleukin-12p70 |
| MCH | Mean Corpuscular Hemoglobin |
| MCHC | Mean Corpuscular Hemoglobin Concentration |
| MCV | Mean Corpuscular Volume |
| NHRC | National Health Research Council |
| NK | Natural Killer |
| NKT | Natural Killer T cells |

| | |
|---|---|
| ORF | Open reading frame |
| PBMCs | Peripheral Blood Mononuclear Cells |
| PCR | Polymerase Chain Reaction |
| PCV | Packed Cell Volume |
| RBC | Red Blood Cells |
| RNA | Ribonucleic Acid |
| RT-PCR | Reverse Transcription- Polymerase Chain Reaction |
| STIDH | Sukraraj Tropical and Infectious Disease Hospital |
| TNF | Tumor Necrosis Factor |
| WHO | World Health Organization |

# ABSTRACT

Dengue fever (DF) is a major global health issue with an unknown immunopathogenesis which is caused by the infection with any of the four closely related serotypes of dengue virus (DENV), transmitted mainly by *Aedes aegypti* mosquito. In tropical and subtropical regions, DF is endemic, worldwide, and disease severity is becoming more prominent. There is a need for biomarkers that can predict and explain DF susceptibility and prognosis. Our study demonstrated the serum cytokine profile in the longitudinal study of the DV-infected patients. Twenty-nine samples were included, out of them sixteen were of dengue virus infected patients and thirteen of them were healthy controls. Six cytokines, namely IL-6, IL-8, IL-10, TNF, IL-12p70, and IL-1β, were measured in serum using a Cytometric Bead Array (BD Bioscience, USA) and a FACS Calibur-E3318 Flow Cytometer System. When compared to the convalescent phase, the levels of IL-6, IL-8, IL-10, and IL-1β in serum of dengue infected patients were significantly higher during the acute phase. Likewise, significant difference was found in the levels of lymphocytes, eosinophils, monocytes, platelets count, PCV and RBC between acute and convalescent phase. These observations with dengue infection provides an insight to understand the immunopathogenesis of dengue and predict the possible biological parameters for dengue fever which can act as the diagnostic, prognostic and therapeutic agents.

**Keywords:** Dengue fever, Immunopathogenesis, Cytokines, Flow Cytometry

# 1. INTRODUCTION

## 1.1 Background

In regions characterized by a tropical or subtropical climates, the *Aedes* mosquito is the primary vector of the most prevalent arthropod-borne viral disease, dengue. The prevalence of dengue has risen up 30 times more in recent years as a result of a combination of factors, including a faster rate of growth of population, global warming, unplanned and unmanaged urbanization, ineffective control of mosquito, travel, and inadequate health care amenities (Hasan et al., 2016). Dengue virus (DENV) has the potential to induce mild or asymptomatic dengue fever (DF), severe dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), both of which can be fatal (Sounsravally et al, 2014). Approximately 400 million dengue infections occur annually, affecting 2.5 billion people, with a mortality rate exceeding 5-20% in some areas. (Hasan et al, 2016). Although approximately fifty percent of the world's population is vulnerable to dengue and the disease was first detected more than 70 years ago, neither a vaccine nor viable therapies are now available. There are a number of reasons why there isn't a "cure" for dengue, including the gaps in our comprehension of dengue immunopathogenesis, the absence of relevant animal representation that would replicate patients' clinical signs, and the intrinsic hazards of giving live attenuated dengue vaccines to individuals lacking antibodies against it (Puc et al, 2021).

### 1.1.1 Dengue Virus

A single-stranded, positive-sense RNA virus with an envelope is known as the dengue virus (DENV). It is a member of the Flaviviridae family and genus Flavivirus. Three structural proteins (core, membrane associated, envelope) and seven nonstructural (NS) proteins are encoded by the DENV genome, which is about 11 kb long. The NS proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Four DENV serotypes exist: DENV-1, DENV-2, DENV-3, and DENV-4. These serotypes are closely related antigenically and share 65% to 70% of the amino acid sequence (Nonyong et al, 2018). Any one of these infections has the potential to provide a lifetime immunity to that particular serotype. A serious illness could be brought on by a novel serotype infection.

Symptoms of dengue infection include a high fever, frontal headache, vomit, fatigue, pain in the joints, and macular rash on the skin, and as many as sixty percent of infections caused by dengue are self-limited. Acute hemorrhagic fever caused by dengue (DHF), multi-organ failure, and a condition called dengue shock syndrome (DSS) are among the fatal consequences that some people may experience (Rijal et al., 2021). Dengue is primarily transmitted by Aedes mosquitos, particularly the mosquito species *Aedes aegypti*, *Aedes albopictus*, and *Aedes polynesiensis*. *Aedes aegypti* is the common major vector, but *Aedes albopictus* and *Aedes polynesiensis* can also serve as transmission vectors depending upon the geographic area (Malavige et al, 2004).

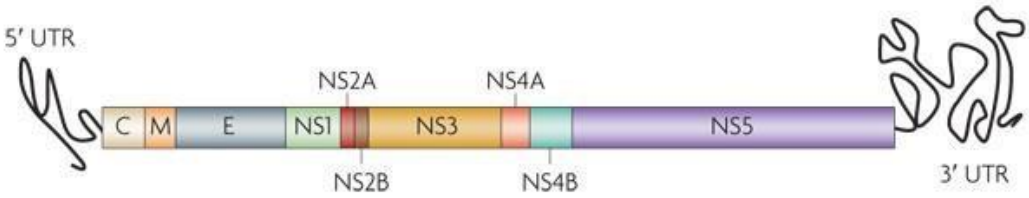


**Figure 1.1:** Genome Sequence of Dengue Virus (Guzman et al, 2010)

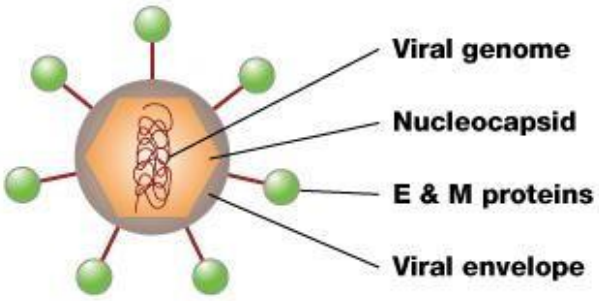


**Figure 1.2:** Morphology of the Dengue Virus (Guzman et al, 2010)

### 1.1.2 Dengue Illness

The clinical syndromes brought on by dengue viruses are diverse, varying from no symptoms to a limited feverish condition and advancing to severe dengue, a potentially fatal state marked by enhanced capillary permeation and shock (Whitehorn and Farrar, 2010). Dengue virus infection has a 4-7 days incubation period (Wang et al 2022). According to WHO's 1997 dengue guideline, infection of DENV can cause anything covering asymptomatic stages, mild febrile episodes, and evolving into

severe dengue complications. Dengue severity varies according to the age of the infected person, the serotype/genotype of DENV, immunity level, and the genetic make-up of the population (Priyadarshini et al, 2010). DF is an acute febrile illness that causes bones, muscles, and joint pain, headaches, leukopenia, and a rash. DHF's four main clinical symptoms are acute fever, hemorrhage usually accompanied by hepatomegaly, and, in serious cases, circulatory collapse. Some infected people could get hypovolemic shock due to significant plasma leakage (Wang et al, 2020). In addition, the 1997 criteria for defining DHF and DSS cases was overly complex to implement in environments with limited resources, overly precise, and missed a significant portion of severe DF cases, such as those involving hepatic failure and encephalitis (CDC, 2019). In 2009, the World Health Organization (WHO) released updated guidelines for diagnosis and management, leading to modifications in case definitions. Currently, there are three categories: dengue fever (DF), dengue fever with warning signs (DFWS), and severe dengue (SD). (Warkentien et al, 2016).

**Table 1.1:** Classification of Dengue Severity and Case Management

| **Dengue without Warning Signs** | **Dengue with Warning Signs** | **Severe Dengue** |
|---|---|---|
| Any patient who has traveled to or lives in a dengue-endemic area and presents with fever (typically 2–7 d in duration) and at least one among the subsequent: | Any patient who meets the criteria for dengue without warning signs and, typically around the time of defervescence, has at least 1 of the following: | Any patient that fits the dengue criteria, with or without warning signs and presents at least one of these symptoms: |
| Nauseousness | Severe stomachache or soreness | Extreme plasma leakage resulting in shock or the collection of fluid outside blood vessels leading to breathing problems. |

| | | |
|---|---|---|
| | | Profuse bleeding from the gastrointestinal tract or vagina necessitating medical intervention like intravenous fluid resuscitation or blood transfusion. |
| Emesis | Continuous vomiting | |
| | | Critical organ dysfunction such as transaminase levels, ≥1000 IU/L, altered consciousness, or heart dysfunction. |
| Skin Irritation | Clinical extravascular fluid accumulation | |
| Soreness and discomfort (headache, eye pain, muscle ache or join pain) | Postural drop in blood pressure | |
| Positive tourniquet test | Mucosal hemorrhage | |
| Low white blood cell count | Lethargy/restlessness | |
| | Liver swelling | |
| | Gradual increase in hematocrit (ie, hemoconcentration) concurrent with rapid decrease in platelet count | |
| Case Management | | |
| Outpatient management | Hospital or observation admission | ICU admission |

Occasionally occurring in patients, dengue hemorrhagic fever (DHF) presents with high body temperature, hemorrhagic tendency, profuse thrombocytopenia and leakage of plasma from vessels. DHF differs from DF due to plasma leakage, which is also the main factor contributing to severity and may cause circulatory failure and death. (Srikiatkhachorn et al, 2017). Around the time of defervescence, leakage of plasma in DHF is frequently observed in the chest and gastrointestinal cavities. Although only 2% to 4% of subsequent DENV infections result in DHF, numerous prospective studies

have revealed an elevated risk of DHF ranging from15 to 80 times higher than that of initial dengue virus exposure. DHF is also strongly associated with secondary DENV infections (Burke et al, 1988).

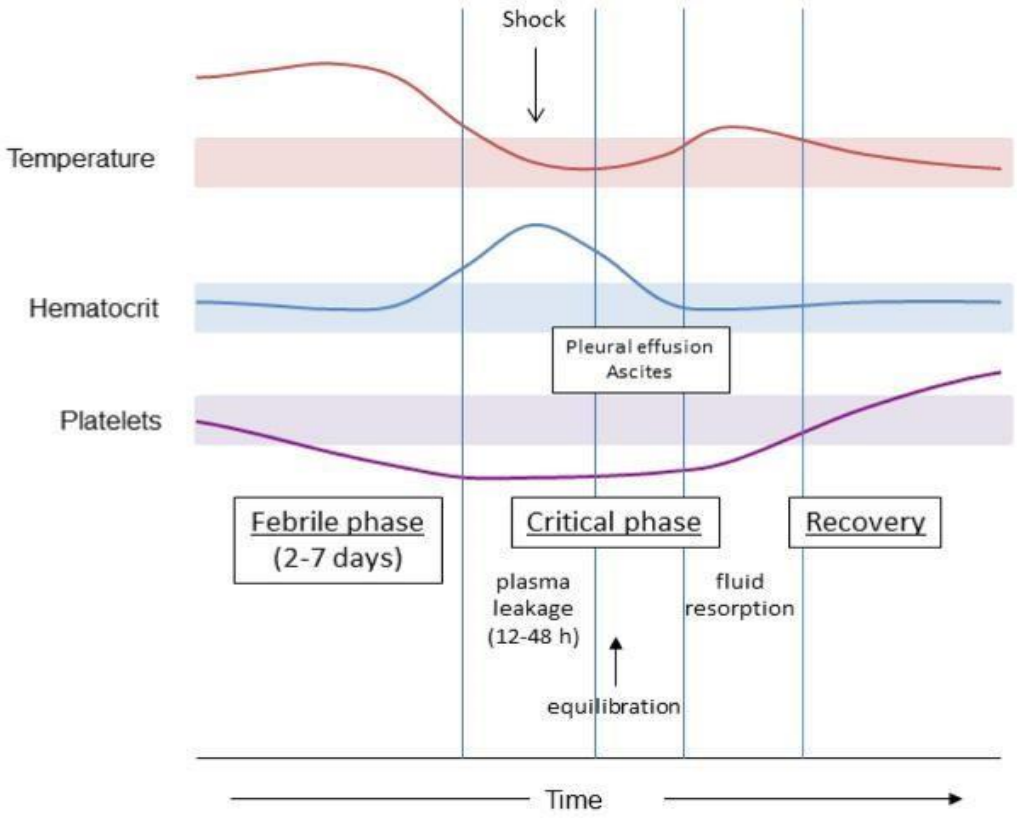


**Figure 1.3:** Clinical course of dengue hemorrhagic fever.

The febrile phase, critical phase, and recovery phase of an illness are shown along with medical events and laboratory findings. Indicative of plasma leakage, a hemoconcentration (an increase in hematocrit) takes place during the critical phase. The illness causes a decline in platelet counts, which reaches their lowest point right when plasma leakage starts. The temperature, hematocrit, and platelet count normal ranges are represented by the shaded areas. (Srikiatkhachorn et al, 2017).

### 1.1.3 Laboratory Diagnosis of Dengue Illness

DF should be taken into account in patients who frequently exhibit a sudden onset of fever, headache, pain in their bodies, and occasionally an allergic reaction spreading from the trunk and who have lived in or have lately moved to the region where the disease is endemic in the two weeks prior to symptom onset. By identifying the pathogen, viral genetic material, markers, antibodies against DENV or a combination

of these approaches, the diagnosis can be made. DENV infection can be identified in the early stages of the illness from serum, plasma, blood cells, or other tissues by detecting viral RNA with nucleic acid amplification tests, NS1 protein with some commercial tests, and isolating the virus in mammalian or mosquito cell cultures to conduct additional genotyping and lineage analysis for comprehensive virus characterization (Muller et al, 2017).

Reverse transcription-polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assays (ELISA), or quick point-of-care tests would be used to detect the viral RNA sequence in serum samples from patients in the acute stages of DF disease (7 days after the start of the illness). The qualitative detection of DENV IgM antibodies using the IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) begins 4-5 days after the onset of symptoms and is also consistently detectable for about 12 weeks. Anti-human-IgM antibody is used in the MAC-ELISA to capture human IgM antibodies on a microtiter plate, and then antigens of DENV crafted from the envelope proteins found in the four serotypes of DENV are added. Patients with IgM positivity undergo plaque reduction neutralization tests (PRNTs), which aim to identify particular neutralizing antibodies targeting DENV as well as other types of flaviviruses, in order to identify the source of the infection or rule out other flaviviruses like ZIKV and YFV, as well as, in some cases, the DENV serotypes that are infecting the patient (WHO, 2009)

**Table 1.2:** Laboratory Tests for Identifying Dengue Infection (WHO,2009)

| Clinical Sample | Diagnostic Approach | Methodology | Time to Results | |
|---|---|---|---|---|
| Virus and virus product detection | Acute serum (1–5 d of fever) and necropsy tissue | Virus isolation | Mosquito or mosquito cell culture inoculation | 1 wk or more |
| | | Nucleic acid detection | RT-PCR and real-time RT-PCR | 1–2 d |
| | | Antigen detection | NS1 Ag rapid test | Minutes |
| | | | NS1 Ag capture ELISA | 1 d |

| Clinical Sample | Diagnostic Approach | Methodology | Time to Results | |
|---|---|---|---|---|
| | | | Immunohistochemistry | 2–5 d |
| Serological response | Paired sera<br>• S1: acute serum from 1–5 d<br>• S2: convalescent serum 15–21 d | IgM or IgG seroconversion (S1 to S2) | ELISA<br>HI | 1–2 d |
| | | | Plaque reduction neutralization test | >7 d |
| | Serum after day 5 of fever | IgM detection | MAC-ELISA | 1–2 d |
| | | | IgM rapid tests (lateral flow) | Minutes |
| | | IgG detection | IgG ELISA<br>HI | 1–2 d |
| | | | IgG rapid tests (lateral flow) | Minutes |

### 1.1.4 Prevention against Dengue Illness

The only method of prevention for dengue is vector control because there is currently no effective vaccine (Hossain et al, 2021). The three fundamental components of preventive and control mechanisms are physical control, biological control, and chemical control. Societal-based control initiatives are examples of physical control techniques. For maximum control of the vector population through community involvement, a number of strategies can be combined. Education campaigns, cleaning initiatives, and removing unwelcome water-collecting habitats in bottles to stop larvae breeding are some of the programs that aim to reduce the number of mosquito breeding grounds. All of these initiatives aid in educating people about the best ways to stop the reproduction of eggs and larvae that later turn into disease-carrying vectors. The use of biological organisms, such as the Wolbachia bacteria, which disrupts the sexual cycle of Aedes mosquitoes, is used to control pest populations (Niang et al, 2018). Additionally, the ongoing use of sterile insect technique, also known as STI, aids in the suppression of mosquitoes that spread serious infections like hemorrhagic dengue fever. The basic idea behind this technique is to disperse male

mosquitoes that have been sterilized in a lab among the targeted population. When these male mosquitoes are released, they help to lower the female mosquitoes' rate of reproduction, which lowers the number of vectors in urban areas (Niang et al, 2018). Utilizing larvivores fish and crustaceans, which assist in lowering vector larvae populations, is another biological control strategy as they eat mosquito larvae and pupae, which can stunt the growth of vectors. The simplest way to eliminate and control dengue fever vectors is through chemical control. To ward off and eradicate Aedes mosquitoes and other insects that spread fatal diseases, chemical agents such as insecticides are used (Swaminathan, 2023).

### 1.1.5 Management of Dengue Illness

Dengue treatment focuses on symptom management. Despite the fact, that, various medications have been investigated as potential dengue therapeutics, none have demonstrated a decrease in viral presence, clinical symptoms, or adverse outcomes (Wong et al, 2022). The preferred treatment after the febrile phase is fluid replacement and antipyretic therapy with paracetamol. Use caution when taking any additional nonsteroidal anti-inflammatory medications. Prudent hydration serves as the cornerstone of therapy during the infection's critical stage. Normal saline, Ringer's Lactate, and 5% glucose diluted 1:2 or 1:1 in normal saline, plasma, plasma substitutes, or 5% albumin are the fluids that are typically administered (Hasan et al, 2016). In December 2015, the CYD-TDV Dengue vaccine received its license. The age range for those who can use it is 9 to 45. It is a live, recombinant vaccine that requires three doses to be effective. It currently has international licenses in twenty countries (Banerjee et al, 2022).

## 1.2 Statement of Problem

Dengue virus infection emerges as a highly consequential viral illness on a global scale, with an estimated 100 million symptomatic cases reported each year, resulting in around 10,000 fatalities (Dhenni et al, 2021). In context of Nepal, Dengue fever outbreaks have occurred on an annual basis since 2006. The most recent data from Nepal's Ministry of Health and Population (11th December 2022) show a total of 54232 cases and a total of 67 deaths have been confirmed to date (Banerjee et al,

2022). Numerous factors can lead to dengue infection in humans, according to some epidemiological studies, which also show that the infection can become severe. Among them is age. Other variables include the host's genetic make-up, the vector's role in the transmission of the disease, the virus's serotype, the gender and genotype of the infected individuals, the environment, their immune systems, the population's socioeconomic status, and secondary infection by a heterologous serotype (Araf et al, 2021). Dengue deaths and casualties are on the rise. However, at present, there are no authenticated clinical or laboratory markers that can reliably forecast the onset of severe disease, such as DHF, DSS, and others. As a result, it is critical to identify the biological parameters that will aid in early prediction of cases that will eventually progress to a severe form and can be used as diagnostic, prognostic and therapeutic agent.

Although several potential risk factors for the development of severe dengue (SD) have been put forth, we still do not fully comprehend the pathogenesis of SD. A cytokine storm, which is a phenomenon that alters cytokine and chemokine levels and causes impairment of endothelial cells operation that eventually result in augmented vascular permeability of endothelial cells leading to plasma leakage, a hallmark of DHF and DSS, is one theory on how SD progresses. (Dengue Shock Syndrome) (Chaturvedi et al, 2000). Cytokines and chemokines are cell signaling molecules that work together to coordinate a number of processes, including immunity, cell proliferation, development, differentiation, and maturation. (Kurane et al, 1994). According to several studies, elevated cytokine levels are linked to altered vascular permeability, leakage of plasma, coagulopathy, and thrombocytopenia (Martina et al., 2009). Thrombocytopenia, leakage of plasma, vascular permeability changes, and altered hepatic transaminase levels have all been linked to IL-10. (Green and Rothman, 2006). Endothelial cells exposed to TNF-α increased permeability and cell death (Lopez et al, 2012). Additionally, TNF-α stimulates the production of tissue factor (TF), which can start the coagulation pathway. As a result, plasma leakage and coagulopathy—the two primary pathological processes in severe dengue—may be significantly influenced by TNF-α (Srikiatkhachorn et al, 2017). Neutrophil infiltration induced by high concentrations of IL-8 locally, results in edema formation because of endothelial

damage caused by neutrophils and subsequently leakage of plasma (Harada et al, 1994). IL-6, CXCL10, CXCL11, and RANTES are secreted by the endothelial cells of DENV infected patients. (Dalrymple et al, 2012). These mediators have chemoattractant properties and can increase permeability, which may contribute to inflammation and plasma levels (Kelley et al, 2012). IL-1β is a powerful pro-inflammatory cytokine associated with fever induction and an inducer of nitric oxide (NO) synthesis in various cells (Geller et al, 1995). NO operates as a signaling messenger, managing vascular function, recruitment of cells, and the aggregation of platelets (Pinheiro et al, 2022).

Inflammatory cytokines like those mentioned above promote and fuel the inflammatory response in many infectious and parasitic diseases. IL-10 demonstrates anti-inflammatory attributes, such as the ability to inhibit inflammatory responses, the presentation of antigens, and phagocytosis. However, the function of anti-inflammatory cytokines, such as IL-10, is crucial in mitigating and maintaining a balance against the potentially harmful activities of inflammatory cytokines. According to Tsai et al. (2013), in some cases, IL-10 may have an immunosuppressive function that results in reduced ability of the immune system to clear the infection, causing a prolonged infectious period during acute viral infections, which may contribute to DENV pathogenesis. Determining the profiles and stability of important inflammatory and anti-inflammatory cytokines during DENV infection is therefore essential for understanding the cellular and molecular pathways that underlie the onset of disease.

Numerous studies have identified a variety of cytokines, such as IFN-, TNF-, IL-6, IL8, and IL-10, as indicators of the severity of dengue. Nevertheless, despite Nepal being one of the dengue hotspots, very few studies, as such, have been conducted there. Therefore, in order to identify the potential biological marker, the immunological profile of the Nepalese patient who has DENV infection is necessary.

## 1.3 Hypothesis

### 1.3.1 Null hypothesis

There is no significant difference in the level of serum cytokines (IL-8, IL-1β, IL-6, IL10, TNF and IL-12p70) in DENV infected patients during acute and convalescent phase, between DF and healthy control.

### 1.3.2 Alternative Hypothesis

There is significant difference in the level of serum cytokines (IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70) in DENV infected patients during acute and convalescent phase, between DF and healthy control.

## 1.4 Objectives

### 1.4.1 General Objective

To study the cytokine storm in a longitudinal study of dengue patient by flow cytometry **1.4.2 Specific Objectives**

- To compare the level of serum cytokines ((IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70) in acute, convalescent and healthy controls
- To compare the clinical parameters in acute and convalescent phase.
- To analyze the correlation between the clinical measures and cytokine responses in DENV infection

## 1.5 Rationale of Study

Dengue is one of the emerging infectious tropical diseases in Nepal. Nepal Government has taken various initiatives to combat the dengue outbreak. However, it is challenging to stop this disease's quick emergence and spread because there are currently no approved therapeutics or vaccines for it. Similarly, there is no dependable predictive test to forecast the onset of severe dengue. A better understanding of both virus genetics and host-immune response are significant for clear insight in dengue pathogenesis. Our study aims to study the immune response of the dengue infected patients and predict the possible biological parameters for dengue fever which can act as the diagnostic, prognostic and therapeutic agents. Additionally, the study aims to build the infrastructure and strengthen local capabilities for future comprehensive immunological studies on dengue and other infectious diseases

# 2. LITERATURE REVIEW

## 2.1 Global Burden of Dengue

Dengue fever is an evolving infectious disease of global significance, with 2.5 to nearly 4 billion people living in regions with a high prevalence of dengue, where an estimated 50-100 million infections occur each year (Warkentein et al,2016). It has never been defined when DENV first emerged in human populations, owing to the fact that the disease is frequently without symptoms and thus goes undiagnosed. Dengue fever was first documented in a Chinese medical encyclopedia in 992 BC (Gubler et al, 1998). Furthermore, sporadic outbreak of a particular illness with a close resemblance to dengue happened in Asia and the Americas before the end of the 18th century; thus, there is a hypothesis that the virus circulate throughout the tropical and subtropical regions between the 19th and 20th centuries (Holmes et al, 2003). The spread of effective mosquito-borne illness across the tropical and subtropical world has been critical to the rise of dengue as a major public health issue. The primary vector, urbanadapted *Aedes aegypti* mosquito, has spread to regions within the tropics and subtropics. It originated in Africa during the slave trade from the 15th to the 19th centuries, expanded across Asia via trade interactions during the 18th and 19th centuries, and has disseminated worldwide in the last 50 years with increased travel and trade (Mousson et al, 2005). In recent years, the geographical distribution of a secondary vector, *A. albopictus*, has widened dramatically. (Lambrechts et al, 2010). The international market expansion, particularly the trade of tyres from used vehicles, has been suggested to be responsible for the dissemination of eggs and undeveloped stages of these arboviral carriers in fresh territories. In Asia and Latin America, endemicity has also been made easier by rapid urbanization, which has led to higher population densities and a plethora of locations for vector proliferation in densely populated urban areas and the areas around them. Although little is known about dengue infections in Africa, recent outbreaks raise the possibility that the disease could spread more widely there. In order to determine the actual burden of diseases, more surveillance is required. (Cameron et al, 2012).

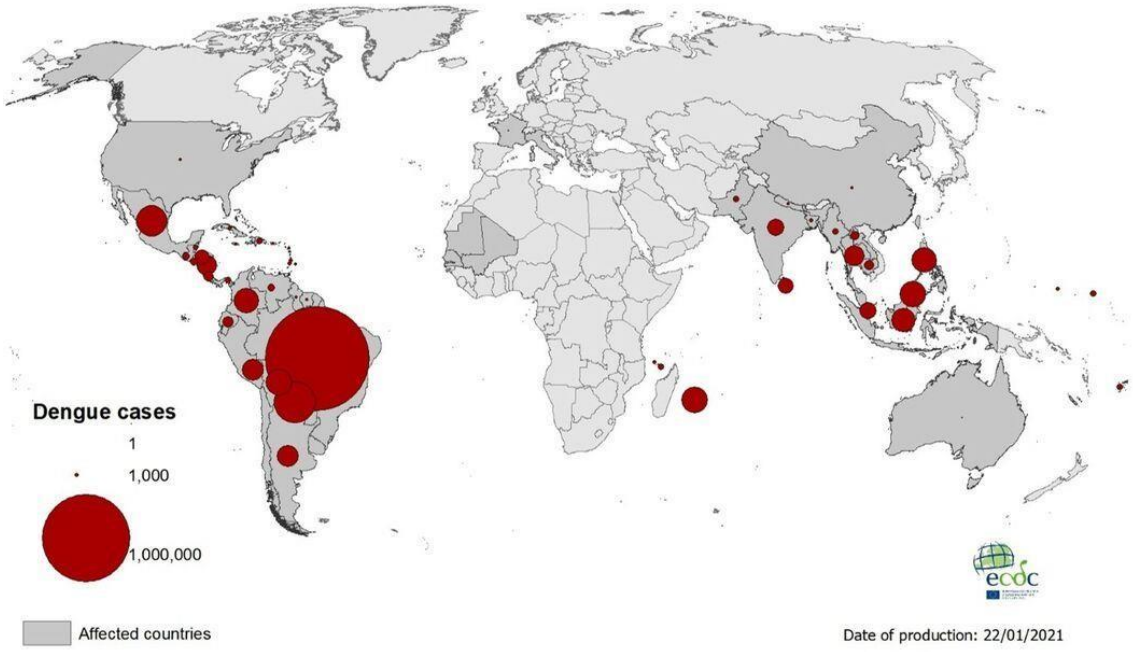


**Figure 2.1:** Geographical distribution of dengue worldwide (ECDC, 2021)

## 2.2 Epidemiology of Dengue in Nepal

Dengue fever was first disclosed in Nepal in 2004 in a Japanese traveler (Pandey et al, 2004) and the study conducted by Takasaki et al., confirmed it to be Genotype 1 of dengue of DENV-2 (Takasaki et al, 2008). Following its initial facade in 2004, Nepal encountered sequence of dengue surges ranging from localized regional spread to widespread national outbreaks (Poudyal et al,2021; Dumre et al; 2013). A cyclical pattern can be seen in the significant large-scale outbreaks that occurred in 2010, 2013, 2016, 2017, and 2019. (Pandey et al, 2022). The COVID-19 pandemic significantly overtook dengue reporting after the historic 2019 outbreak with 17,992 cases (Pandey et al, 2021; Tun et al, 2021). This phase served as a foreword to an unusual catastrophe, and in 2022, the most extensive dengue outbreak in Nepal's history took place, with an explosive increase in events from August 8 to August 26 (WHO, 2022), despite sporadic cases being observed as early as January. According to the Epidemiology and Disease Control Division, there were 54,784 cases and 88 deaths because of dengue cases extending throughout Nepal's 77 districts. The largest dengue epidemic in Nepal was due to DENV-1 and DENV-3, with DENV-2 being a minor serotype. This is the first time that three DENV serotypes have been confirmed in Kathmandu (Rimal et al, 2022). Despite significant outbreaks in Nepal in 2006, 2010,

2013, 2018, and 2019, there remains a scarcity of information regarding the circulating DENV serotypes within the country (dominant serotypes: DENV-1/2, DENV-2, DENV-1, DENV-2, and DENV-2/3 in 2010, 2013, 2016, 2017, and 2019 respectively) (Dumre et al, 2013; Dumre et al, 2020; Ngwe et al, 2021; Prajapati et al, 2020). This indication suggests that various serotypes circulate and that serovar shift incidents occur between iterative dengue surges in Nepal (Rimal et al, 2022).

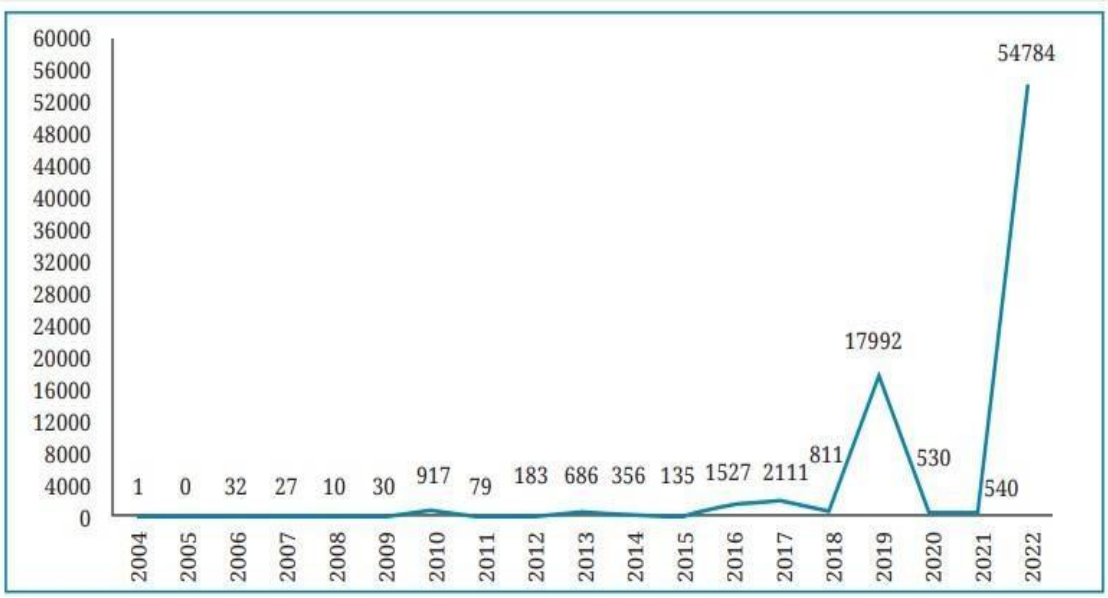


**Figure 2.2:** Annual trajectory of dengue occurrence in Nepal from 2004 to December 31, 2022 (EDCD, Ministry of Health and Population, Nepal)

## 2.3 Pathophysiology of Dengue

Dengue virus infection and manifestations of critical dengue is extremely complicated and its pathogenesis is poorly understood. DHF/DSS is indicated by leakage of plasma and abnormal hemostasis, but its exact mechanism remains unknown despite being recognized for five decades (Sellahewa et al, 2013). The fact that the DENV infection appears in its worst stage when the virus is being eliminated by the immune response of the host, rather than at peak virus load, provides support to the idea that the immune reaction of humans is important in the pathogenesis of disease. (Green et al, 2006). Among the proposed theories on dengue immunopathogenesis are (i) the antibody enhancement theory (Halstead et al, 1970), (ii) cross-reactive memory T cell activation (Kurane et al,1992), and (iii) the original antigenic sin (Mongkolsapaya et al, 2003), all of which cause either excess production or a distorted profile of cytokine

expression, giving rise to the term's cytokine storm/cytokine tsunami. By increasing the permeability of capillaries and causing leakage, the cytokines storm directly impacts endothelial cells in the vascular system. (Mathew et al, 2008). Cytokines can also act synergistically, like when tumour necrosis factor-alpha (TNF-), interferon- (IFN-), and interleukin-1 (IL-1) interact to enhance the permeability of capillaries in contrast to when a particular cytokine acts alone. (Bukre et al, 1993).

The antibody dependent enhancement (ADE) of infection theory is the most commonly accepted elucidation for the emergence of DHF and DS. This theory states that patients are more likely to develop DHF and/or DSS if they have preexisting DENV antibodies and contract a secondary infection with DENV that is caused by an entirely distinct serotype of DENV rather than the initial infection. Additional factors, such as virological, immunological, and host-specific elements have recently been shown to perform significant roles in the the progression of severe DENV disease (Katzelnick et al., 2017; Pang et al., 2007), despite the fact that ADE is central to the predominant theory for explaining various kinds of severe DENV disease.

DHF is characterized by increased vascular permeability, which is linked to elevated levels of cytokines associated with inflammation such as TNF-, IL-1beta, IL-6, IL-8, IL-12, and anti-inflammatory cytokines such as IL-10 (Fink et al, 2006; Green & Rothman, 2006; Kittigul et al, 2000; Srikiatkhachorn et al, 2017). According to a study by Mehta VK et al., dengue patients with neurological symptoms had significantly upregulated serum and CSF concentrations of IL-6 and IL-8 than controls (Mehta et al, 2017). According to a different study, DHF cases levels of IL-6 and IL-8 was significantly elevated than DF cases did. Because IL-8 levels were associated with thrombocytopenia, elevated alanine transaminase (ALT), and thrombocytopenia, IL-8 levels appeared to be more relevant to the pathogenesis of DHF (Priyadarshini et al, 2010). Other research has suggested that cytokine levels can be used to predict dengue severity (Bozza et al, 2008; Srikiatkhachorn et al, 2017). Yet, the physiological roles of cytokines can vary (Salazar-Mather et al, 2000); therefore, it is essential to study the control of diverse cytokines secreted by DV-infected individuals.

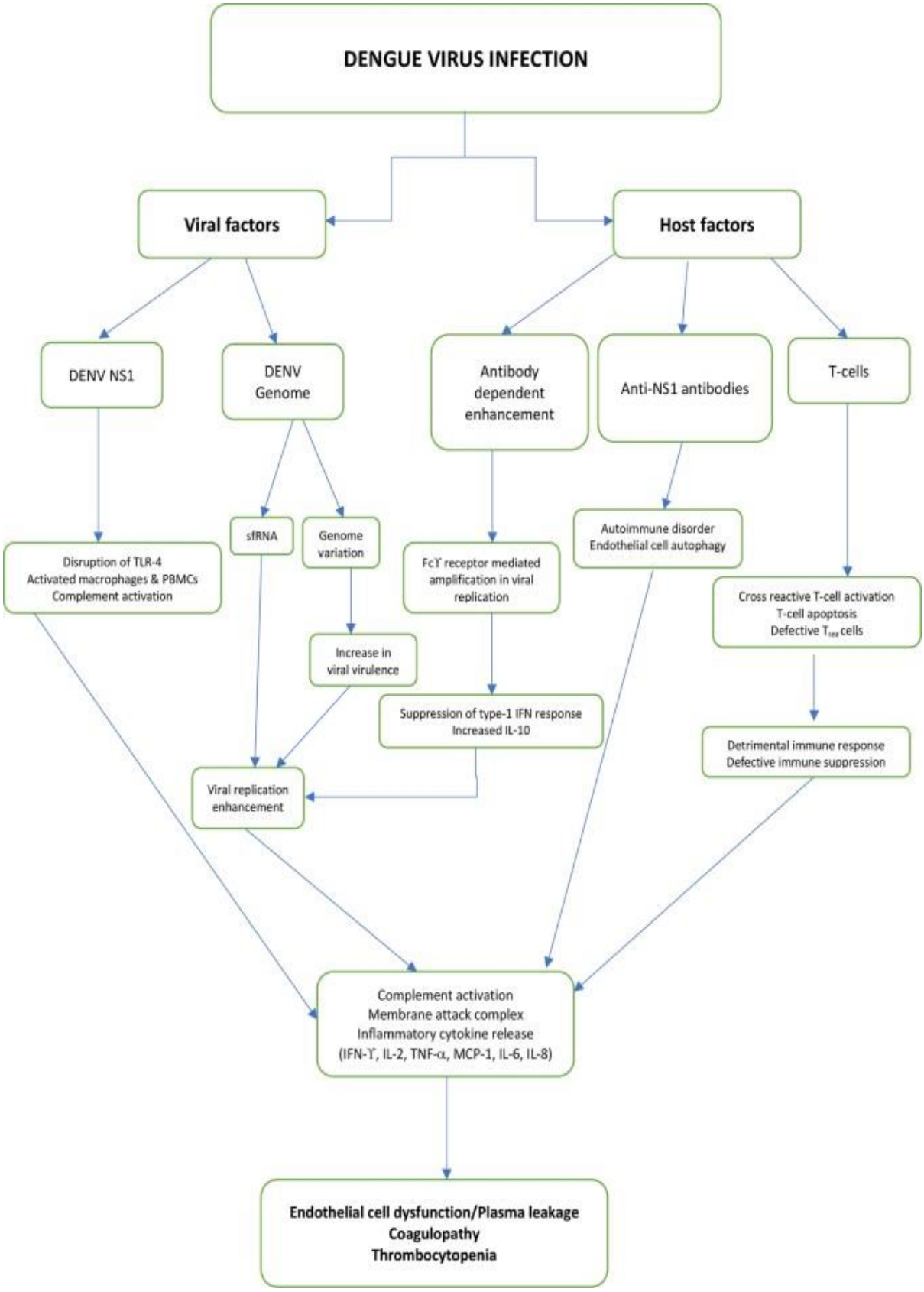


**Figure 2.3:** Complex interconnection of viral and host elements in the progression of dengue virus infection (Bhatt et al, 2021)

## 2.4 Cytometric Bead Array (CBA)

The development of flow cytometric bead-based technology has given researchers a fresh technique for measuring numerous analytes synchronously in biological and environmental samples. Fluorescent bead array assays, such as the Cytometric Bead Array™ (CBA; BD Biosciences, San Jose, CA), have greatly expanded the assay dynamic range. This novel innovation allows for the faster estimation of numerous samples on a sole platform, the evaluation of a number of analytes in one sample, taking advantage of small amounts of samples to obtain data, uniformity and outcomes comparable to previous experiments, and straightforward comparison with existing assays. The cytometric bead array (CBA) system measures multiple analytes at the same time in sample volumes that are too small for traditional immunoassays. The results of an analysis of several human cytokines have been presented. Furthermore, the technology enables the design and development of assays for a wide range of analytes, such as inflammatory agents, chemotactic factors, immunoglobulin subclasses, intracellular signaling components, apoptosis regulators, cell adhesion molecules, and antibody classes (Morgan et al, 2004).

BD CBA assays present an approach to capture a soluble molecule or collection of soluble molecules utilizing beads characterized by size and fluorescence, facilitating the identification of these molecules through flow cytometry. Every individual capture bead within a BD CBA kit is associated with a specific antibody. The detection reagent in the kit comprises antibodies conjugated with phycoerythrin (PE), producing a fluorescent signal corresponding to the quantity of the bound analyte. When the unknown sample, containing identified analytes, is incubated with the capture beads and detection reagent, it leads ti the formation of sandwich complexes (consisting of capture bead, analyte, and detection reagent). Using flow cytometry, these complexes can be measured to find particles that exhibit both bead- and detector-specific fluorescence properties.

The BD CBA Human Inflammatory Cytokine Kit makes utilized bead-based array technology to concurrently identify numerous cytokine proteins present in research samples. The proteins IL-1β, IL-6, IL-8, IL-10, TNF, and IL-12p70 were the targets of capture antibodies that were coated on six bead populations with varying

fluorescence intensities. The bead array is created by combining the six bead populations. In order to create sandwich complexes for the assay, the beads for trapping cytokines are combined with synthetic standards or unidentified samples and incubate the mixture with marker antibodies that have been PE-conjugated. After collecting samples on a flow cytometer, the level of PE fluorescence emitted by each sandwich complex indicates the concentration of the cytokine.

## 2.5 Cytokines

Cytokines are proteins that function as inflammatory mediators or modulator molecules. They are secreted during innate and adaptive immune responses. They include interleukins (IL), tumor necrosis factors (TNF), chemotactic cytokines, interferons (IFN), mesenchymal growth factors, transforming growth factor, and various additional cytokines and growth factors (Zhang and An, 2007). Cytokines work together to coordinate a number of processes, including immunity, cell growth, proliferation, differentiation, and maturation (Kurane et al., 1994).

### 2.5.1 Interleukin -10

The cytokine synthesis inhibitory factor IL-10, which is primarily secreted by monocytes, was found to be a homodimer of 178 amino acids with a signal sequence of 18 amino acids and a mature segment of 160 amino acids with a molecular weight of about 18 kDa (monomer) (Zdanov et al., 1995).

Pe'rez and colleagues (2004) demonstrated that the people infected with dengue during the 1997 epidemic in Cuba had elevated IL-10 levels, with secondary infections being the cause of more pronounced levels. According to Kuczera et al. (2018), among secondary infections, DHF cases had higher IL-10 levels than DF cases. According to Libraty et al. (2002), plasma leakage and serum IL-10 levels in dengue are correlated. Patro et al. (2019) discovered a substantial rise in the level of IL-10 in both severe and non-severe dengue. IL-10, a substance with immunosuppressive properties, stimulates the production of SOCS3, which blocks the signaling pathways induced by IL-6, IL-2, IL-12, IFN-a, IFN-g, and nuclear factor-kappa B, enabling viruses to evade immune surveillance (Flores-Mendoza et al, 2017).

Malavige et al. (2012) demonstrated a negative correlation between IL-10 levels and T cell counts, emphasizing the role of IL-10 in accelerating the progression of disease by impairing T cell response. High levels of IL-10 and viral NS1 protein levels were found to be positively correlated in the serum of people with acute dengue infection by Adikari et al. (2016). The authors also showed that high levels of IL-10 are produced by monocytes but not T cells in cultures when NS1 is present. According to Tsai et al. (2013), IL-10 was found in high levels in ADE models with DENV-2. Azeredo et al (2006), Chunhakan et al. (2015), Laur et al. (1998), Zhao et al. (2016), noted elevated IL-10 in DHF/DSS patients.

### 2.5.2 Interleukin-8

The only chemokine referred to as IL was identified in 1987 as a neutrophil chemotactic factor called IL-8. As of the new chemokine nomenclature, CXCL8 has replaced IL-8 as the name for the substance. It belongs to the ELR + CXC chemokine family and promotes angiogenesis and neutrophil recruitment as its main biological effects (Waugh & Wilson, 2008; Yoshimura, 2015). The majority of Il-8, contains 99 amino acids and is secreted by monocytes and macrophages (Brat et al., 2005).

IL-8 plays a variety of roles in the body, including those related to angiogenesis, lymphoid trafficking, wound healing, and inflammation. This cytokine might contribute to the IFN-g antiviral potential in viral infections (Zhou et al., 2018). According to Y. Huang et al. (2000) and Bosch et al. (2002), these activities are essential for drawing neutrophils, naive T cells, basophils, and eosinophils to the infection site. Additionally, according to Medin et al. (2005), IL-8 causes the release of leukotrienes, lytic enzymes, platelets-activating factors, and a burst of neutrophils that breathe in order to protect the host from pathogens that are invading. The production of IL-8 is promptly triggered by an array of stimuli, encompassing pro-inflammatory cytokines like TNF, IL-1, IL6, and IFN-gamma (Brat et al., 2005), as well as bacterial and viral products (Medin et al., 2005). Normally, non-induced cells hardly secrete IL-8. In vitro research has demonstrated that IL-8 is produced by a diverse range of cell types, encompassing monocytes, macrophages, neutrophils, NK cells, and somatic cells such as endothelial cells, fibroblasts, and epithelial cells (Juffrie et al, 2000).

Infection of human PBMC with DENV-2 in vitro resulted in an increase in IL-8 expression (Moreno-Altamirano et al., 2004). Medin et al. obtained a similar result in 2005 when experiments conducted in vitro with human myeloid or endothelial cells infected by DV showed an elicitation of IL-8 secretion. Furthermore, it was suggested that the NS5 protein of DENV2 virus contributes to the initiation of IL-8 expression and thus is capable of counteracting antiviral effects of intrinsic immunity (especially IFNa) and thus promote viral disseminating to bystander cells that were not infected. Yang et al. (2016) claimed that cross-serotype secondary infection with DENV-2 in PBMC of DENV-1-infected subjects stimulated higher IL-8 production than homologous secondary infection.

Human PBMC infected in vitro with DENV-2 experienced an increase in the expression of IL-8(Moreno-Altamirano et al, 2004). Resembling conclusions were found by Medin et al. in 2005, who reported that in vitro infection of human myeloid or endothelial cells with DV stimulates the release of IL-8. They put forward that the viral NS5 protein of DENV2 plays an integral part in triggering IL-8 expression and is capable of reversing the antiviral effects of innate immunity (especially IFNa), which in turn promotes viral spreading to bystander uninfected cells. In contrast to a homologous secondary infection, Yang et al. (2016) asserted that higher IL-8 production was induced by heterologous secondary DENV-2 infection in PBMC from DENV-1-infected individuals. All of these discoveries highlighted the significance of this chemokine in the host's fight against the DV infection. Nevertheless, a different finding showed that DF patients' levels of IL-8 were not significantly higher than those of normal people (Huang et al., 2000). Additionally, King et al. (2002) demonstrated that the human mast cell/basophil line infected with DV did not exhibit an elevated level of IL-8. Juffrie et al. (2000) discovered an increase in IL-8 levels in dengue cases than the control subjects. Many later findings suggested that individuals infected with DENV had higher levels of IL-8 than healthy controls, and disease with serious cases had elevated levels of IL-8 than the individuals with mild cases (Cui et al., 2016; N. Pandey et al., 2015; Patra et al., 2015). Chen and Wang (2002) discovered that DV-infected monocytes/macrophages produce IL-8. Furthermore, DENV-1 seems to cause the most elevated levels of IL-8 among the various serotypes. (Feitosa et al., 2016).

### 2.5.3 Interleukin-1β (IL-1β)

IL-1 family cytokines are known as 'early-response' cytokines since they are secreted at the beginning of the immune defense mechanism and function as the starting point for a subsequent cascade of cytokines that are proinflammatory. The interleukin-1 family is made up of several pro- and anti-inflammatory proteins, with IL-1β being the best-known pro-inflammatory member (Dinarello, 2018). Monocytes and macrophages are the major cellular sources of IL-1β. The proinflammatory actions of IL-1β are attained by triggering their respective receptors on the cell surface, namely the IL-1β type 1 receptor (IL-1RI) and the IL-18 receptor chain (IL-18R) (Zhang & An, 2007).

High levels of IL-1β have been observed to manifest during dengue. According to Tuyen et al. (2020), infected people produce more of this cytokine than uninfected people do. As a result of cellular apoptosis (Jaiyen et al, 2009), IL-1β levels are also higher among the patients diagnosed with DHF than patients diagnosed with DF (Cui et al., 2016; Kamaladasa et al., 2016). The difference in IL-1β levels between dengue patients and healthy people, however, were not different, according to research carried out by Arias et al. (2014).

### 2.5.4 Interleukin 12-p70 (IL-12p70)

IL-12p70, which is a member of the Interleukin-12 family of cytokines, is a heterodimeric 70 kDa cytokine made up of a 197 amino acid (p35) and a 306 amino acid (p40) subunit. Monocytes and macrophages are the primary producers of it in response to agents that are infectious, and it significantly affects the number of Th1 cells and Th1-type cytokines. Th1-type cytokines are upregulated by IL-12p70, whereas Th2-type cytokines are more prevalent in its absence (Romani et al., 1997).

According to de la Cruz Hernández et al. (2016), dengue patients had higher levels of IL-12p70 than normal individuals. According to Kuczera et al. (2018), in vitro infection of human monocytes resulted in increased IL-12 secretion. Furthermore, human monocyte-derived macrophages infected with DENV were used in an ADE model to create a heterologous secondary infection in PBMC in vitro, which resulted in elevated levels of IL-12p70 (Kamaladasa et al, 2016; Yang et al, 2016). IL-12p70 levels were

elevated in DF patients than in DHF patients, according to research by Pacsa and his colleagues from the year 2000. The authors also noted that as the severity of the disease increased, those levels progressively decreased. (Pacsa et al., 2000). Additionally, Arias and colleagues showed that severe disease cases were connected to lower IL-12p70 levels, attributing a protective role to this cytokine (Arias et al, 2014). According to Fagundes et al. (2011), nitric oxide is required for IL-18 and IL-12p70 to promote IFNg production and regulate host resistance to dengue.

### 2.5.5 TNF

TNF-alpha, also known as tumor necrosis factor, was discovered in 1975 as an endotoxin-induced glycoprotein that causes hemorrhagic necrosis sarcomas in mice after sarcoma transplantation. Monocytes and macrophages under the influence of infectious agents produce it primarily as a a protein that is 233 amino acids long and weighs 17 kDa. (Kriegler et al, 1988; Tang et al, 1996).

According to Jaiyen et al. (2009) and Torrentes-Carvalho et al. (2009), TNF-alpha is a proinflammatory cytokine that is directly associated with apoptosis of cells, which has an antiviral function and may be connected to how severe the illness is in DENV infections. Patients with dengue infection have higher levels of TNF-alpha than healthy individuals do (Feitosa et al., 2016), and patients with severe disease have higher levels of TNF-alpha than DF people do (Cui et al, 2016; Kittigul et al, 2000; Zhao et al, 2016). According to Imad et al. (2020) TNF alpha was significantly higher in case of severe hepatitis. However, other studies confirmed that patients with DHF and DF are identical. (Laur et al, 1998; Malavige et al, 2004; Senaratne et al, 2016). In vitro studies revealed that DENV-2 infection increased TNF-alpha expression and production in human PBMC (Torrentes-Carvalho et al., 2009). An ADE induction model using human monocyte-derived macrophages revealed a similar phenomenon (Kamaladasa et al., 2016). Hatch et al. (2011) show how the advancement of dengue may exhibit nonlinearity. When investigating intracellular cytokine production in CD4+ and CD8+ T cells of children infected with DENV in a secondary infection scenario, it was found that subjects who developed subclinical secondary infection had a greater proportion of DENV-specific TNF-alpha, IFN-gamma, and IL-12-producing cells in comparison to symptomatic cases (Hatch et al., 2011). Additionally, it was discovered that human

PBMC from heterologous secondary infections produced more TNF than those from homologous secondary infections (Sierra et al., 2012; Yang et al., 2016). However, the ethnicity of the DENV-infected patient may affect TNF-a production (Restrepo et al., 2008). Sam et al. (2015), on the other hand, examined a group consisting of 196 cases of DHF/DSS patients and discovered a link between TNF-alpha alleles that are highly expressed and protection from DHF/DSS. According to the authors, by accounting for the infection's time course, these elevated TNF-alpha levels might achieve their protective properties by lowering DENV load at the initial stage of the infection by taking into account the infection's time course.

### 2.5.6. Interleukin-6

Il-6 is a member of the IL-6 family and has a molecular weight range of 21 to 28 kDa, 212 amino acids, a signal peptide of 27 amino acids, and two potential NH2-linked glycosylation sites, using the gp130 receptor as a signaling subunit (Cronstein, 2007; Tanaka et al., 2014). IL-6 is a multifunctional cytokine with many immunological activities that can cause an acute phase response (Rachman & Rinaldi, 2006). IL-6 is primarily produced by monocytes and macrophages, with minor contributions from connective tissue cells, blood vessel lining cells, T lymphocytes, B lymphocytes, cartilage cells, and skin cells (Cronstein, 2007).

In vitro studies revealed that infection of human macrophages and B lymphocytes with DENV-2 or DENV-3 could trigger the production of IL-6 (Lin et al, 2002). Furthermore, infection of human monocytes with DENV-4 significantly elevate this cytokine more than infection with other serotypes (Levy et al, 2010). The expression and production of IL-6 were increased in ADE models after infection with human monocyte-derived macrophages (Kamaladasa et al, 2016). Additionally, human macrophages infected under ADE conditions and treated with anti-IL-6 had higher IFN- levels. As a result, IL-6 is believed to play a direct part in the suppression of the antiviral reaction and higher viral load (Rolph et al, 2011). Pinto et al. (1999) demonstrated that IL-6 levels were noticeably higher in dengue patients than in controls (Pinto et al., 1999). Chen demonstrated a significant increase in serum IL-6 levels in adult dengue patients (L.-C. Chen et al., 2006). Furthermore, Feitosa discovered healthier people have higher plasma levels of IL-6 than patients with dengue (Feitosa et al, 2016), whereas Levy

discovered that Dengue patients have higher levels of IL-6 than healthy people. (Levy et al., 2010). Several studies have found that DHF patients have higher IL-6 levels than DF patients. However, there was no difference between patients who experienced primary or secondary infections or between those who experienced shock (Brasier et al, 2012; Malavige et al, 2012; Priyadarshini et al, 2010; Restrepo et al, 2008). However, Butthep found enhanced presence of this cytokine in DSS patients, and Restrepo discovered that children with secondary infection had significantly higher levels of IL-6 (Restrepo et al., 2008). Chen and Wang, on the other hand, were unable to detect IL-6 in DV-infected monocytes/macrophages (Y.-C. Chen & Wang, 2002). Additionally, according to Priyadarshini et al. (2010), all dengue cases had IL-6 levels that were not noticeably higher than those of healthy controls.

# 3. METHODS AND METHODOLOGY

## 3.1 Ethical approval

The Nepal Health Research Council (NHRC) and the Central Department of Biotechnology (CDBT) Research Committee provided their ethical approval for this study. The privacy of the samples was maintained. In case of children, sample was collected after the guardian filled the consent form on the behalf of children.

## 3.2 Site selection

The Sukraraj Tropical and Infectious Disease Hospital (STIDH) in Kathmandu is where the samples were obtained, in the three months' time duration from October-December.

For Convalescent, the sample was collected in the three months' time interval.

## 3.3 Clinical case confirmation

The study included patients who had an acute febrile illness lasting one to seven days and exhibited dengue fever symptoms. For the purpose of identifying dengue cases, the WHO, 2009 guidelines were strictly followed. Only those participants who had a fever and accompanied by two of these symptoms—nauseousness, vomiting, rashes, headache, and malaise—were included in the study. Demographic information like age, sex and address, symptoms and diagnosis were recorded by attending physicians in the Study CRF (Case report form).

## 3.4 Sample collection and storage

### 3.4.1. Sample size

During 2022 dengue outbreak, paired acute and convalescent serum samples were collected. Serum was acquired both during the acute episode and during the convalescent period, from days 2 to 15 and days 21 and over after the onset of fever, respectively. In this study, 29 volunteers were involved out of which 16 were acute DV infected and 13 were healthy control. Also, 16 convalescent samples were collected from the dengue infected patient enrolled in the study during acute phase infection.

### 3.4.2 Suspected cases

The serum was extracted from the blood samples and kept at -80°C until it was used. The samples were divided into three groups based on how severe they were: Dengue without Warning Signs (Fever with two of the following symptoms: nausea, vomiting, rashes, muscles and joints pain), Dengue with Warning Signs (abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, or liver enlargement >2 cm), and Severe Dengue (severe bleeding, shock, organ failure, or fluid accumulation with respiratory distress) (WHO, 2009).

### 3.4.3. Healthy control

A total of thirteen healthy control samples were included in this study. The healthy controls were selected as people who did not show any symptoms of illness. The healthy controls were used for the study of change in serum cytokine level with respect to dengue and non-dengue cases.

### 3.4.4. Hematological parameters

The hematological tests: Platelets count, hematocrit, hemoglobin were performed using automated machine (Sysmex XN-300), for AST) for all the dengue suspected cases at the sites where the samples were collected. The details of the clinical parameters have been shown in Appendix 2 and 3 for all the enrolled cases.

### 3.4.5. Sample inclusion and exclusion criteria

The sample collection criteria were according to WHO case definition 2009. The subjects must have fever accompanied by any pair of the following symptoms: nausea, vomiting, rash, aches and pains. Samples were not collected from the patients who did not consent for our study. Also, samples were not taken from children under 2 years old, pregnant individuals and HIV infected patients.

## 3.5 Cytokine measurement by cytometric bead array (CBA) assay using flow cytometry (BD FACS Caliber)

The FACS Calibur-E3318 Flow Cytometer System facilitated the assessment of IL-1,

IL-6, IL-8, IL-10, IL-12p70, and TNF- were measured using a Cytometric Bead Array (BD Biosciences, USA) following the guidelines provided by the manufacturer.

### 3.5.1 Human Inflammatory Cytokines Standards Preparation

One lyophilized vial of Human Inflammatory Cytokine Standards was transposed to a 15ml polypropylene tube labelled "Top Standards." Two ml of Assay Diluent was added on it and then incubated for 15 minutes at room temperature then gently mixed by pipette. Eight polypropylene tubes were labeled arranged in following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. 300ul of Assay Diluent was pipetted in each tube and serial dilution was performed. 300ul of top standard was transferred to the 1:2 dilution tube and carefully agitated by pipette. Serial dilution was continued by transferring 300ul from 1:2 tubes to 1:4 tubes and so on to 1:256 tubes. Negative control was prepared with Assay Diluent only.

### 3.5.2 Human Inflammatory Cytokine Capture Beads Preparation

Each capture bead suspensions were vigorously vortex. Fifteen millilitre polypropylene tube was labeled “Mixed Capture Bead” in which 10 ul per sample of each capture bead was added and mixed by vortex. Supernatant from the tube was carefully aspirated and discarded after being centrifuged at 400g for 5 minutes. The mixed capture beads pellet was thoroughly vortexed while suspended in the serum enhancement buffer.

### 3.5.3 Sample Preparation

Dengue patient serum sample stored at -80°C was thawed at -20°C followed by 4°C and allowed to equilibrate at room temperature.

### 3.5.4 Human Inflammatory Cytokine Assay

Mixed capture bead was vortex and 50ul was added in each assay tubes. As shown in the following table, 50 ul of the human Inflammatory Cytokine Standard dilutions were added to the control tubes.

| Tube Label | Concentration (pg/ml) | Standard Dilution |
|---|---|---|
| 1 | 0 (Negative Control) | Assay Diluent Only |
| 2 | 20 | 1:256 |

| | | |
|---|---|---|
| 3 | 40 | 1:128 |
| 4 | 80 | 1:64 |
| 5 | 156 | 1:32 |
| 6 | 312.5 | 1:16 |
| 7 | 625 | 1:8 |
| 8 | 1250 | 1:4 |
| 9 | 2500 | 1:2 |
| 10 | 5000 | Top standard |

Fifty ul of each unknown sample were added to a polypropylene tube with a label. Then, 50 ul of the Human Inflammatory Cytokine Phycoerythrin Detection Reagent was added to all assay tubes and carefully mixed the tubes to resuspend the pellet and they were left to incubate at room temperature for 3 hours while being shielded from light. After incubation 1ml of wash buffer was added to each tube and centrifuged at 400g for 5 minutes. Supernatant was cautiously removed from each assay tubes. Then 300 ul of Wash Buffer were introduced into each assay tube.

### 3.5.5 Acquisition of captured cytokine and Fluorescence measurement

After cytometer setup, the captured cytokines were acquired. First standard cytokines were acquired followed by unknown sample acquisition.

### 3.5.6 Calculation of Cytokine Concentration

The calibration curve created using the standard for each cytokine was used to determine the intensity of cytokines in each unknown sample.

## 3.6 Statistical Interpretation of Data

The Mann-Whitney U test was employed to compare demographic and clinical characteristics. When paired acute and convalescent phase dependent samples were compared, the cytokine concentrations were expressed in pg/ml, and Mann-Whitney U test was used for two independent samples. Correlation coefficients were calculated with Spearman's test. Results were analyzed using Graph pad PRISM 9.0, and the data were considered significant at $P<0.05$.

# 4. RESULTS

## 4.1 Demographic information of patients infected with DV

Dengue samples were collected from the Sukraraj Tropical and Infectious Disease Hospital. Out of 16 dengue cases, seventy- one (75%) of them were males and twenty-nine (25%) were females, with an average of 33 years. The characteristics of study population are shown in Table 4.1.

**Table 4.1:** Demographic information of patients infected with DV

| Parameters | Number (%) |
|---|---|
| Total number of Dengue Cases | 16 |
| Male | 12 (75%) |
| Female | 4(25%) |
| Mean age (Years ±SD) | 33±15 |

## 4.2 Clinical Signs and Symptoms of DV-Infected Patients

In our study, the sign and symptoms seen in dengue infected patients were fever, bodyache, lethargy, nausea, vomiting, abdominal pain and other symptoms such as eye-pain, cough, itchy-throat, diarrhea, etc. Fever was presented by 100% of the DV-infected patients. Similarly, body ache such as muscle, joint- pain, nausea, vomiting, abdominal pain and other symptoms were presented by 50%, 56.25%, 62.5%, 31.25%, 31.25% and 23.53% of the total DV-infected sample patients respectively.

**Table 4.2:** Clinical Manifestations of Patients with DV Infection

| Manifestation of illness | Total reported DF cases (%) |
|---|---|
| Fever | 100 |
| Body ache (Muscles, bone) | 50 |
| Lethargy | 56.25 |
| Nausea | 62.5 |
| Vomiting | 31.25 |
| Abdominal Pain | 31.25 |
| Other symptoms (eye-pain, cough, itchy throat, diarrhoea) | 23.53 |

## 4.3 Dengue Infection Categories and Hematological Parameters

A significant difference was found in the level of lymphocytes, eosinophil, monocytes, platelet count, PCV, RBC between acute and convalescent phase using Wilcoxon test.

**Table 4.3:** Comparison of hematological findings in Dengue infection during acute and convalescent phase

| **Variable** | **Acute (mean± SD)** | **Convalescent (mean± SD)** | **P value** |
|---|---|---|---|
| Haemoglobin (Hb) (gm/dl) | 14.7±1.5 | 14.4±1.5 | 0.3039 |
| Total count (TLC)/cumm | 6622.5±3910.6 | 7576.8±2172.9 | 0.1474 |
| Neutrophils(%) | 61.7±12.4 | 55.6±8.7 | 0.0805 |
| Lymphocytes (%) | 25.5±10.5 | 32.5±8.1 | 0.0346* |
| Eosinophils (%) | 1.8±2.2 | 3.25±2.1 | 0.021* |
| Monocytes (%) | 11±2.6 | 8.6±1.5 | 0.0055** |
| Basophils (%) | 0 | 0 | 0 |
| platetes count (*1000/cumm) | 174.3±77.6 | 265.6±119.7 | 0.0003*** |
| PCV (%) | 43.2±4.4 | 41.7±4.4 | 0.0146* |
| RBC (million/cumm) | 5.1±0.7 | 4.9±0.8 | 0.0043** |
| MCV(%) | 86.3±9.9 | 87.1±9.7 | 0.0869 |
| MCH(%) | 29.7±3.5 | 30.1±3.8 | 0.1719 |
| MCHC(%) | 34.3±0.6 | 34.6±0.7 | 0.3438 |

## 4.4 Comparison of cytokines in serum of DV-infected patient

Wilcoxon test revealed that there is significant elevation in the level of IL-8, IL-1β, IL6 and IL-10 in the serum of DV-infected patient during acute phase than convalescent phase. Similarly, Mann- Whittney U test results showed that there is significant elevation in the level of IL-1β, IL-6, IL-10 and IL-12p70 in the serum collected from individuals with DV infection than healthy controls. Likewise, the level of IL-8 in healthy control is significantly elevated when compared to the convalescent phase which suggest that the patient is still in recovery phase.

**Table 4.4:** The mean concentration (pg/ml) of cytokines in serum of DV-infected patient and healthy controls

| Cytokine | Concentration of serum cytokines in DV-infected patient (pg/ml) | | P value | | HCvs Acute | HC vs Convalescent |
|---|---|---|---|---|---|---|
| | Acute Phase (Mean± SD) | Convalescent Phase (Mean± SD) | | Healthy controls (Mean± SD) | P value | P value |
| IL-8 | 83.22±106.9 | 27.34±6.61 | 0.0002*** | 34.49±9.75 | 0.3291 | 0.0437* |
| IL-1β | 18.82±5.09 | 16.72±4.87 | 0.0182* | 14.54±2.12 | 0.0087** | 0.51 |
| IL-6 | 48.13±46.12 | 22.18±16.08 | 0.0027** | 16.96±2.56 | <0.0001**** | 0.4293 |
| IL-10 | 27.97±19.38 | 14.97±1.44 | <0.0001**** | 16.42±4.35 | 0.0001*** | 0.2729 |
| TNF | 18.94±9.76 | 27.08±49.14 | 0.1711 | 15.92±3.53 | 0.313 | 0.6735 |
| IL-12p70 | 26.21±35.88 | 27.14±43.26 | 0.7531 | 15.55±1.61 | 0.0189* | 0.6114 |

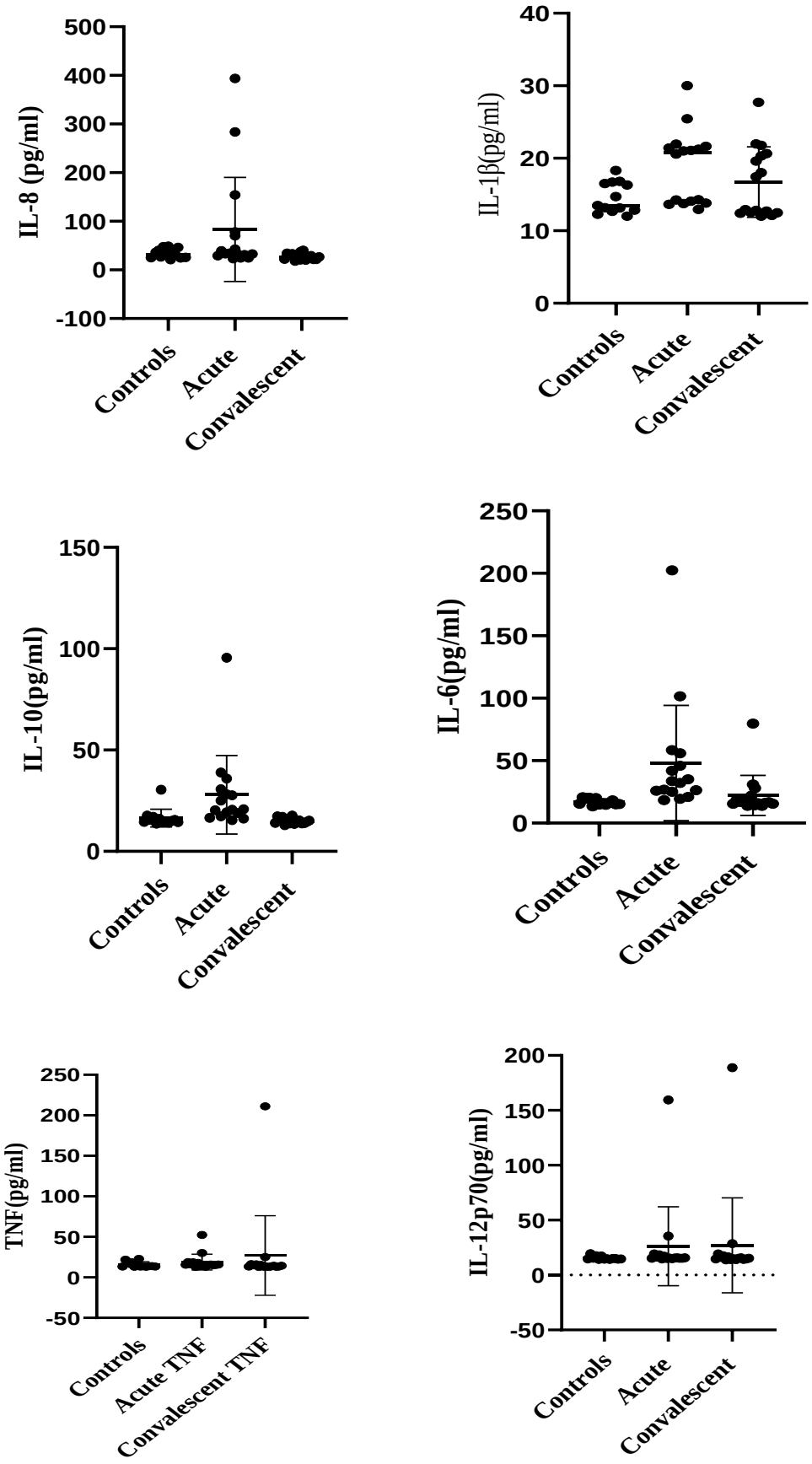


**Figure 4.1:** Comparison of cytokines- IL-8, Il-1β, IL-6, IL-10, TNF and IL-12p70 (in pg/ml) respectively, between healthy controls, acute phase and convalescent phase

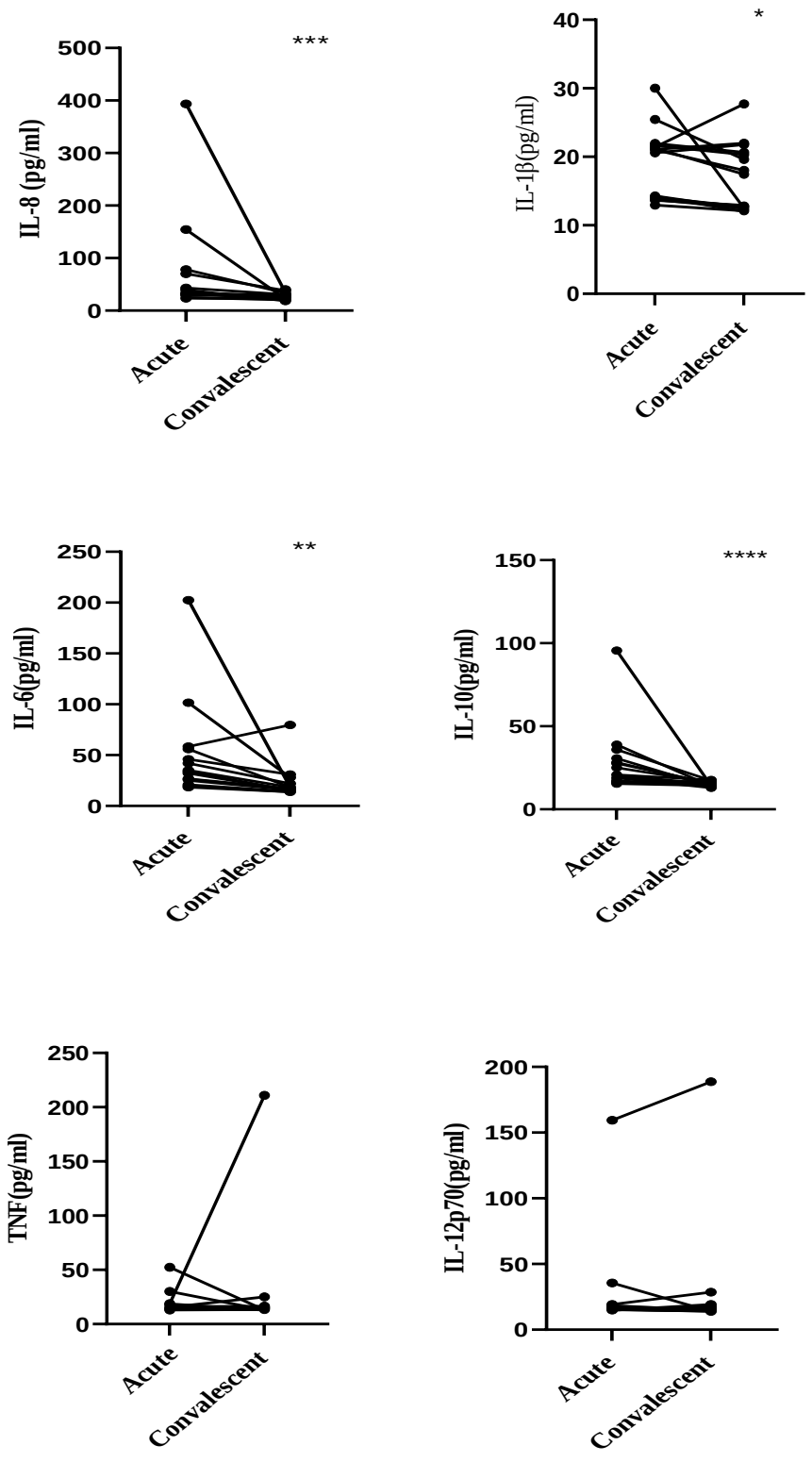


**Figure 4.2:** Correlation study of Cytokine Level of TNF and IL-12p70 (pg/ml) between acute and convalescent phase of DENV infected patient (*$P < .05$, **$P < .01$, ***$P < .001$, *****P<.0001)*

## 4.5 Variances and Associations in Cytokines Levels between Age and Clinical Signs of Disease

The Mann-Whitney U test results showed that there is no substantial difference in cytokine levels across varying age categories (age ≤35 and >35) (Table 4.5). Similarly, there is no significant difference($p<0.05$) between cytokine levels in presence or absence of nausea (Table 4.6), vomiting (Table 4.7) and abdominal pain (Table 4.8). Likewise, there is significant difference in IL-1β level and IL-6 level in presence or absence of lethargy (Table 4.9) and body ache (Table 4.10) respectively.

**Table 4.5:** The mean cytokine concentration (pg/mL) in DV infected patients across varying age classifications (≤35 years and >35 years)

| Cytokines | Mean Cytokine Concentration (pg/ml) in patients with DV infection<br>Age<br>>35<br>(Mean± SD) | <br><br>≤35<br>(Mean± SD) | Mann-Whitney U test<br>P value |
|---|---|---|---|
| IL -8 | 53.33±24.35 | 93.19±122.38 | 0.7703 |
| IL-1β | 21.50±0.39 | 17.93±5.64 | 0.1033 |
| IL-6 | 40.19±13.27 | 50.78±53.12 | 0.5209 |
| IL-10 | 27.18±11.89 | 28.23±21.76 | 0.8615 |
| TNF | 15.71±0.27 | 20.02±11.17 | 0.8418 |
| IL-12p70 | 51.46±71.99 | 17.79±5.76 | 0.7918 |

**Table 4.6:** The mean concentration (pg/mL) of cytokines in DV infected patients in the absent or present of nausea

| Cytokines | Mean Cytokine Concentration (pg/ml) in patients with DV infection<br>Nausea<br>Absent<br>(Mean± SD) | <br><br>Present<br>(Mean± SD) | Mann-Whitney U test<br>P value |
|---|---|---|---|
| IL-8 | 92.38±147.71 | 77.73±82.60 | 0.7128 |
| IL-1β | 18.83±6.49 | 18.82±4.44 | 0.7128 |
| IL-6 | 40.65±31.09 | 52.63±54.29 | 0.5622 |
| IL-10 | 21.97±5.16 | 31.57±23.94 | 0.5622 |
| TNF | 24.37±14.92 | 15.69±1.95 | 0.1896 |
| IL-12p70 | 19.36±8.08 | 30.31±45.39 | 0.9371 |

**Table 4.7:** The mean cytokine concentration (pg/mL) in DV infected patients for both vomiting and non-vomiting groups

| Cytokines | Mean Cytokine Concentration (pg/ml) in patients with DV infection | | Mann-Whitney U test P value |
|---|---|---|---|
| | Vomiting | | |
| | Absent (Mean± SD) | Present (Mean± SD) | |
| IL-8 | 69.71±108.13 | 112.96±109.49 | 0.8269 |
| IL-1β | 17.88±3.98 | 20.90±7.04 | 0.4409 |
| IL-6 | 32.35±11.90 | 82.85±73.69 | 0.1451 |
| IL-10 | 23.24±7.90 | 38.36±32.50 | 0.2674 |
| TNF | 17.13±4.57 | 22.93±16.62 | 0.9799 |
| IL-12p70 | 28.81±43.35 | 20.48±8.49 | 0.0962 |

**Table 4.8:** The mean cytokine concentration (pg/mL) in patients with DV infection, with or without abdominal pain

| Cytokines | Mean Cytokine Concentration(pg/ml) in patients with DV infection | | Mann-Whitney U test P value |
|---|---|---|---|
| | Abdominal pain | | |
| | Absent (Mean± SD) | Present (Mean± SD) | |
| IL-8 | 93.31±125.20 | 61.03±108.13 | 0.3196 |
| IL-1β | 19.71±5.46 | 16.86±3.93 | 0.3773 |
| IL-6 | 56.57±53.97 | 29.58±8.36 | 0.3196 |
| IL-10 | 30.20±23.09 | 23.05±5.6 | 0.7427 |
| TNF | 19.39±11.10 | 17.96±6.89 | 0.6804 |
| IL-12p70 | 30.53±43,18 | 16.69±1.59 | 0.6023 |

**Table 4.9:** The mean cytokine concentration (pg/mL) in patients with DV infection, with or without lethargy

| Cytokines | Mean Cytokine Concentration (pg/ml) in patients with DV infection | | Mann-Whitney U test P value |
|---|---|---|---|
| | Lethargy | | |
| | Absent (Mean± SD) | Present (Mean± SD) | |
| IL-8 | 132.18±145.87 | 45.15±41.54 | 0.0712 |

| | | | |
|---|---|---|---|
| IL-1β | 21.88±1.64 | 16.45±5.66 | 0.0229* |
| IL-6 | 42.36±11.97 | 52.62±61.88 | 0.1142 |
| IL-10 | 24.39±9.45 | 30.74±24.86 | 0.8371 |
| TNF | 16.19±1.19 | 21.08±12.88 | 0.7007 |
| IL-12p70 | 36.10±54.39 | 18.51±6.57 | 0.9795 |

**Table 4.10:** The mean cytokine concentration (pg/mL) in patients with DV infection, with or without body ache

| **Cytokines** | **Mean Cytokine Concentration (pg/ml) in patients with DV infection** | | **Mann-Whitney U test** |
|---|---|---|---|
| | **Body ache (Muscle, Joints)** | | **P value** |
| | **Absent (Mean± SD)** | **Present (Mean± SD)** | |
| IL-8 | 79.28±92.65 | 86.29±122.27 | 0.8371 |
| IL-1β | 20.91±3.49 | 17.2±5.71 | 0.0712 |
| IL-6 | 66.26±61.15 | 34.03±25.94 | 0.0229* |
| IL-10 | 35.74±27.81 | 21.92±5.52 | 0.2523 |
| TNF | 15.82±1.72 | 21.38±12.69 | 0.8966 |
| IL-12p70 | 36.59±54.18 | 18.13±6.67 | 0.2865 |

## 4.6 Correlations among the Cytokine Profiles and Clinical Manifestations

As demonstrated in table 4.11, spearman rank order correlation indicates a robust and positive relationship between the IL-1β level and both hemoglobin ($p<0.001$, $r=0.7647$) and PCV ($p<0.01$, $r=0.7497$). Similarly, the level of IL-6 is positively and moderately correlated ($p<0.05$, $r=0.55$ and $r=0.5259$) with TLC and Lymphocytes respectively. On the other hand, TNF is negatively and moderately corelated ($p<0.05$, $r=-0.5348$) with MCV and positively and strongly correlated ($p<0.01$, $r=0.7082$) with the level of RBC. The level of IL-8, IL-10 and IL-12p70 did not show significant correlation with the level of hematological findings. Likewise, the level of IL-6 is positively and strongly correlated ($p<0.05$, $r=0.6265$) with IL-1β (Table 4.12). However, there was no significant correlation among the other cytokines.

**Table 4.11:** Spearman rank order correlation between the cytokines and hematological findings

| Category | Spearman rho value ® | | | | | |
|---|---|---|---|---|---|---|
| | **IL-8** | **IL-1β** | **IL-6** | **IL-10** | **TNF** | **IL-12p70** |
| Hb(gm/dl) | 0.2147 | 0.7647*** | 0.3029 | -0.09118 | 0.3841 | 0.2077 |
| TLC(/cumm) | -0.3853 | 0.1265 | 0.55* | -0.08824 | 0.07506 | 0.3225 |
| Neutrophils(%) | -0.3267 | 0.06181 | 0.4665 | 0.1163 | -0.2931 | 0.1695 |
| Lymphocytets(%) | 0.2349 | -0.1182 | -0.5259* | -0.1123 | 0.3252 | -0.0858 |
| Eosinophils(%) | 0.1529 | -0.1654 | -0.2933 | -0.05617 | -0.04685 | -0.8175 |
| Monocytes(%) | 0.3447 | 0.4941 | 0.2234 | 0.08876 | 0.2628 | 0.007407 |
| Platelete (*1000/cumm) | -0.1971 | -0.08529 | 0.4265 | -0.1088 | 0.1545 | 0.2518 |
| PCV(%) | 0.1867 | 0.7497** | 0.2815 | -0.06815 | 0.4174 | 0.2203 |
| RBC(million/cumm) | 0.03387 | 0.3564 | -0.06038 | -0.3004 | 0.7082** | 0.3392 |
| MCV(%) | 0.1803 | -0.1271 | -0.08721 | 0.08278 | -0.5348* | -0.339 |
| MCH(%) | 0.2156 | -0.09517 | -0.06394 | 0.05353 | -0.4918 | -0.3165 |
| MCHC(%) | -0.1757 | -0.2176 | 0.09205 | 0.08368 | -0.4229 | -0.3436 |

**Table 4.12:** Spearman rank order correlation among the cytokines

| | **IL-6** | **IL-8** | **IL-10** | **IL-12p70** | **TNF** |
|---|---|---|---|---|---|
| IL-1β | 0.6265* | 0.22353 | 0.3147 | 0.2887 | 0.4735 |
| IL-6 | | -0.02941 | 0.4088 | 0.2887 | -0.04562 |
| IL-8 | | | -0.3176 | -0.243 | -0.04268 |
| IL-10 | | | | 0.2754 | -0.09272 |
| IL-12p70 | | | | | 0.2535 |

# 5. DISCUSSION

Dengue fever is an emerging contagious disease worldwide, with 2.5 to nearly 4 billion people who reside in countries with dengue endemicity, where 50–100 million infections are thought to occur annually (Warkentein et al, 2016). DENV belongs to the Flaviviridae viral family, specifically classified within the Flavivirus genus, and it infects people when an Aedes mosquito bites them, most frequently an Aedes aegypti mosquito (Clyde et al. 2006; Noisakran & Perng 2008; Martina et al. 2009). Any of the four strains of DENV can be contracted and cause a variety of clinical symptoms, from dengue fever (DF), a febrile illness, to dengue hemorrhagic fever (DHF), the most critical state of the disease. Despite the fact that the pathogenesis of DF is still poorly understood, numerous reports indicate that deviations in cytokine response may be crucial in the disease's progression to its worsening stages. (Jadhav et al, 2017). In this study, in an effort to comprehend the immune response mechanism to DV infection, a possible cytokine system and function were put forth. The immune response of DVinfected patients was influenced by several cytokines, which were investigated.

The investigation of the cytokine induction profiles from dengue virus-infected patients during the dengue outbreak in 2022 has been the primary focus of this study. In our study, total 29 samples were included, out of them 16 were of DV-infected patients during acute phase, 16 were of convalescent phase and 13 of them were healthy controls. Six different cytokines were measured: IL-8, IL-6, IL-12p70, TNF, IL-10 and IL- 1β. We studied the changes in various hematological parameters among the various stages of DV-infected patients, along with the changes and associations of various cytokines with these parameters. In the acute phase, we found a significant decrease in the levels of lymphocytes, eosinophils, monocytes, platelets count, PCV and RBC. In the study conducted by Samadi-AI et al. (2021); Jayaratne et al. (2012), similar results were demonstrated. The consequence of the inflammatory process is what causes the decline in eosinophil levels in the acute phase. Low platelet production or increased destruction of platelets through activation of C3 complement protein and additional attachment of the C5b-9 complex to the surface of the platelet may be the cause of the drop in platelets (Ojha et al, 2018). Low RBC levels may make it more likely for dengue virus to trigger the development of transitory polyclonal

antibodies against RBC antigens, which would then cause complement-mediated hemolysis (Kulkarni et al, 2014). IL-6 and IL-1β was found positively correlated indicating that these two cytokines may act synergistically during acute phase of DENV infection. While certain molecules (IL-6, IL-10, and CXCL-10) were observed to remain elevated during the recovery phase in the previous study, in the current investigation, throughout the convalescent phase of dengue infection, the serum concentrations of the majority of cytokines typically reverted to a baseline level. However, in our study, IL-8 levels in the convalescent phase were markedly lower than in the healthy controls. It might be that the patient is still in recovery state.

In this study, age did not significantly correlate with the levels of IL-1, IL-6, IL-8, IL10, IL-12p70, or TNF, suggesting that age do not have any impact on the production of the cytokines. Furthermore, since, no substantial relationship was found between these two groups, the levels of these cytokines were not associated to the abdominal discomfort, nauseousness, or vomiting experienced by dengue patients. However, there was a significant association of IL- 1β with lethargy and IL-6 with bodyache.

In this study, it was found that DV-infected patients had significantly higher serum IL-1beta levels than the healthy control group. The essential proinflammatory cytokine IL1β functions as a pyrogen in the host, raising body temperature. (Kugelberg, 2016). Similar results were reported by Tuyen et al. (2020), Hozz et al. (2013) and Wu et al. (2013) where higher level of IL-1β was observed in the dengue patients compared with healthy individuals. Furthermore, these findings were strengthened by the study carried out by Pan et al. (2020) where they included the samples from DENV patients as well as a DENV infection mouse model. The obtained results demonstrate that both in humans and mice, DENV increases the serum level of IL-1β, strongly indicating that DENV triggers IL-1β in vivo. An earlier in vitro study found that DENV activates the NLRP3 inflammasome to induce the expression of IL-1β in macrophages, and this finding may account for the higher levels of IL-1β in dengue patients compared to healthy controls (Wu et al, 2013).

The levels of TNF- α found in our study were not statistically significant, unlike other research findings that reported increased TNF-α levels in individuals diagnosed with

DHF and DSS (Green et al., 1999; Iyngkaran et al., 1995; Braga et al., 2001). Given the absence of DHF cases, the disease's severity was likely milder in this instance.

Additionally, the TNF-α gene's genetic variation might be a contributing factor (Fernandez et al, 2004; Gupta et al, 2009).

We have demonstrated the significant increase in the level of IL-6 during the acute phase in DV infected patients than healthy controls. This finding aligns with the results documented in the studies conducted by Pino et al. (1999) and Juffrie et al. (2001). Additionally, it has been demonstrated that IL-6, which is generated by macrophages and stimulated endothelial cells, acts as a pivotal facilitator of fever and acute-phase response. Furthermore, IL-6 induces the expression of tissue factors by endothelial cells, which mediates changes in coagulation and fibrinolysis. The overproduction of IL-6 caused by various DENV serotypes is a key factor in the pathogenesis brought on by the dengue virus, and it has been associated with various systemic alterations during an acute inflammatory reaction. Moreover, individuals experiencing severe DENV infections have demonstrated heightened serum concentrations of IL-6 (Coutinho-daSilva etal, 2022).

Notably, earlier research demonstrated a link between increased IL-10 levels and dengue pathogenesis and disease severity (Bhatt et al, 2021). A study by Green et al. (1999) indicates that IL-10 is essential for the emergence of Dengue hemorrhagic fever. According to Libraty et al. (2002), dengue infection is associated with elevated IL-10 levels and platelet deterioration. The function of lymphocytes and platelets may be inhibited by IL-10 (Azeredo et al, 2001). In a study conducted by Petphong et al. (2023), IL-10 levels in secondary infection patients were consistently higher than in the control group. According to Mongkolsapaya et al. (2003), the finding of T cell apoptosis induction during secondary virus infection suggests that IL-10 may be responsible. On the other hand, a potent inflammatory mediator of vascular injury, platelet-activating factor (PAF), may be modulated by IL-10 (Bussolati et al., 2000). In the study conducted by Rathakrishnan et al. (2012), the level of IL-10 significantly decreased towards a healthy level, during convalescent phase. This finding was similar to our results where, we have found the significant elevation of IL-10 in the acute phase of a DENV- infected patients.

We demonstrated the significant elevation of IL-12p70 in the acute phase of DENV infected patient than the healthy controls. This conclusion is supported by a study done by Pacsa et al. (2000), which showed that patients with a less intense form of dengue and in the early stages of illness had activated IL-12p70 genes and increased levels of IL-12p70 proteins. Patients with DHF grades I and II had lower levels of IL-12p70, while those with DHF grades III and IV had no IL-12p70 at all, suggesting that IL12p70 is produced in the initial reaction to dengue viral infection and may play a role in protecting against serious dengue manifestation. Similarly, in the study conducted by New et al. (2022), they demonstrated IL-12p70 were significantly higher in primary infection.

A chemokine called IL-8 that is made by hepatocytes, endothelial cells, and monocytes promotes inflammation, increases vascular permeability, and stimulates the innate immune system. Mangione et al. (2014) discovered that the DSS and DHF groups had notably higher levels of IL-8 than the DF and control groups. IL-8 has been found to be secreted by dengue-infected endothelial cells in vitro (Huang et al, 2000; Bosch et al, 2002). Endothelial cells are important in the progression to severe dengue since their stimulation and fatal injuries cause permeability of the vascular system, leakage of plasma, and hemorrhage. In the study conducted by Pandey et al. (2015), IL-8 concentrations exhibited a substantial elevation in severe dengue cases in contrast to both non-severe dengue cases and individuals from the healthy control group. Parallel findings were noted in studies conducted by Raghupaty et al. (1998) and Juffrie et al. (2000), revealing minimal or negligible levels of IL-8 in individuals afflicted with non-severe dengue. This finding is similar to our study where there is no significant elevation of IL-8 during the acute phase of DENV-infected patient as the population included in the sample was with mild severity of the disease.

# 6. SUMMARY AND CONCLUSION

## 6.1 SUMMARY

A significant emerging infectious disease, dengue viral infections (DVI) have emerged as one of the most significant global infections caused by mosquito-transmitted viruses. Dengue viruses have the potential to induce diverse clinical syndromes, spanning from asymptomatic infection to mild, self-resolving feverish conditions to life-threatening dengue, a potentially fatal condition marked by elevated permeability of capillaries and shock. In context of Nepal, since its first outbreak in 2006, after the first time detection in 2004 from a Japanese traveller, with five successive outbreaks occurring at three year intervals in 2010, 2013, 2016, 2019, and 2022, the infection developed an alarming trend. The prevalence rates were exponentially higher on the previous two outbreaks. It is challenging to stop this disease's quick emergence and global spread because there are currently no approved therapeutics or vaccines for it. The lack of effective treatment options for this disease necessitates early disease detection, early disease monitoring, and an enhanced understanding of disease pathogenesis in order to improve clinical management. This study aims to highlight the immune response of the DENV infected population against the dengue virus and predict the possible biomarker for dengue fever which can act as the diagnostic, prognostic and therapeutic agents.

The serum samples from the dengue infected individual were taken from Sukraraj Tropical and Infectious Disease Hospital (STIDH), Kathmandu. A total of 16 dengue cases with the clinical symptoms that were positive to the rapid diagnostic test and 13 healthy controls were included. The serum sample during convalescent phase was collected at the interval of 3 months form the sample collected during acute phase. In order to study immune response the concentration of different cytokines (IL-6, IL-8, IL-10, TNF, IL-12p70 and IL- 1β) were measured using cytokine bead array kit in flow cytometer and the correlation was established within and with clinical parameters. A significant difference was found in the levels of lymphocytes, eosinophils, monocytes, platelets count, PCV and RBC between acute and convalescent phase. Similarly, there was a significant difference in the level IL-6, IL-10, IL- 1β and IL-12p70 between healthy

control and acute phase of DV-infected patients. Likewise, there was a significant association of IL- 1β with lethargy and IL-6 with bodyache.

## 6.2 CONCLUSION

Our study demonstrated the serum cytokine profile in the longitudinal study of the DV-infected patients. Sixteen paired samples of Acute and Convalescent, and 13 healthy control with total of 29 samples were designed for the research project. In the acute phase, we found a significant decrease in the levels of lymphocytes, eosinophils, monocytes, platelets count, PCV and RBC. The levels of IL- 1β, IL-6, IL-8, IL-10, IL-12p70, and TNF, in this study, did not significantly correlate with age which suggest that age do not have any impact on the production of the cytokines. Besides, the levels of these cytokines did not contribute to abdominal pain, nausea and vomiting that were observed in dengue patients since no significant relationship was obtained between these two groups. However, there was a significant association of IL- 1β with lethargy and IL-6 with bodyache. Serum levels of IL-6, IL-8, IL-10 and IL- 1β were significantly upregulated during the acute phase as compared to convalescent phase. These results provided information for us to understand the interplay between the virus and the host responses during the acute stage of dengue infection. These results not only extended our understanding on the immunological features of the patients in the Nepal dengue outbreak in 2022 but also provide novel insights to the underlying causes of dengue fever in human.

# LIMITATION OF THE STUDY

- The study was carried irrespective to the serotype of the dengue. As a result, the effect of serotype on cytokine profiles was not investigated.
- The cytokine profile between mild and severe dengue patients could not be compared as there were no severe patients included in our sample.
- The sample size in our sample was small.
- Despite the fact that gender differences in dengue severity have previously been described, due to sample size constraints, this study did not evaluate gender. The study's precision could have been hampered by the small sample size.

# RECOMMENDATIONS

For a better understanding of the balance between circulating cytokines and their influence on the progression of severe dengue, additional large prospective studies are recommended.

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# APPENDICES

**APPENDIX 1:** Clinical Symptoms of Dengue Virus Infected Patients

| S.N. | Sample ID | Age | Sex | Fever | Rashes | Headache | Body ache (Muscles, bone) | Lethargy | Hemorrhagic manifestation | Nausea | Vomiting | Abdominal Pain | others symptoms |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 294 | 26 | M | Intermittent | no | Yes | yes | yes | no | Yes | yes | Yes | eye pain, lipoma |
| 2 | 314 | 27 | M | Intermittent | no | Yes | yes | yes | no | Yes | No | No | - |
| 3 | 241 | 20 | F | Intermittent | no | Yes | no | yes | no | Yes | yes | No | - |
| 4 | 282 | 26 | M | Intermittent | no | Yes | yes | yes | no | Yes | No | No | - |
| 5 | 307 | 35 | F | Continuous | no | Yes | yes | yes | no | Yes | No | No | - |
| 6 | 249 | 30 | M | Intermittent | no | No | yes | yes | no | No | No | Yes | - |
| 7 | 97 | 47 | M | Intermittent | no | Yes | no | no | no | No | No | No | - |
| 8 | 242 | 12 | F | Intermittent | no | Yes | no | yes | no | Yes | No | No | - |
| 9 | 245 | 30 | F | Intermittent | no | Yes | yes | yes | no | Yes | yes | Yes | Itchy throat |
| 10 | 160 | 22 | M | Intermittent | no | Yes | no | no | no | No | No | No | - |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 11 | 64 | 74 | M | Intermittent | no | Yes | no | no | no | Yes | yes | No | - |
| 12 | 119 | 31 | M | Continuous | no | Yes | yes | yes | no | No | No | Yes | - |
| 13 | 69 | 44 | M | Continuous | no | Yes | no | no | no | Yes | No | No | - |
| 14 | 65 | 57 | M | Intermittent | no | Yes | no | no | no | No | yes | No | - |
| 15 | 142 | 28 | M | Intermittent | no | Yes | no | no | no | Yes | No | Yes | - |
| 16 | 42 | 23 | M | Intermittent | no | Yes | yes | no | no | No | No | No | Diarrhoea |

**APPENDIX 2:** Hematological Parameters of Dengue Patients During Acute Phase

| **S. N.** | **SAMPLE ID** | **Haemoglobin (Hb) (gm/dl)** | **Total count (TLC)/cumm** | **N (%)** | **L (%)** | **E (%)** | **M (%)** | **B (%)** | **platetes count (*1000/cumm)** | **PCV (%)** | **RBC (million/cumm)** | **MCV (%)** | **MCH (%)** | **MCHC (%)** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 294 | 14.82 | 2790 | 65 | 26 | 1 | 8 | 0 | 69 | 43 | 4.86 | 89 | 31 | 34 |
| 2 | 314 | 16.54 | 11160 | 71 | 18 | 0 | 11 | 0 | 270 | 49 | 5.78 | 84 | 29 | 34 |
| 3 | 241 | 12.23 | 2960 | 56 | 33 | 1 | 10 | 0 | 163 | 36 | 3.22 | 110 | 38 | 35 |
| 4 | 282 | 15.3 | 3310 | 66 | 24 | 0 | 10 | 0 | 114 | 44 | 5.08 | 86 | 30 | 35 |
| 5 | 307 | 12.29 | 7900 | 59 | 31 | 3 | 7 | 0 | 147 | 39 | 5.3 | 74 | 25 | 34 |
| 6 | 249 | 12.95 | 3870 | 39 | 49 | 1 | 11 | 0 | 164 | 39 | 6.33 | 61 | 21 | 33 |
| 7 | 97 | 16.66 | 9840 | 77 | 14 | 0 | 9 | 0 | 125 | 49 | 6.21 | 79 | 27 | 34 |
| 8 | 242 | 12.13 | 17430 | 82 | 9 | 0 | 9 | 0 | 330 | 35 | 4.14 | 85 | 29 | 35 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 245 | 14.49 | 11180 | 72 | 13 | 7 | 8 | 0 | 239 | 42 | 4.37 | 95 | 33 | 35 |
| 10 | 160 | 15.34 | 6950 | 52 | 31 | 5 | 12 | 0 | 331 | 45 | 4.85 | 92 | 32 | 35 |
| 11 | 64 | 13.31 | 3630 | 66 | 23 | 1 | 10 | 0 | 96 | 39 | 4.5 | 86 | 30 | 34 |
| 12 | 119 | 15.71 | 4710 | 67 | 18 | 1 | 14 | 0 | 144 | 46 | 5.1 | 89 | 31 | 35 |
| 13 | 69 | 16.04 | 4960 | 49 | 33 | 1 | 17 | 0 | 145 | 47 | 5.26 | 89 | 31 | 34 |
| 14 | 65 | 16.53 | 4090 | 35 | 43 | 6 | 15 | 0 | 89 | 49 | 5.26 | 93 | 31 | 34 |
| 15 | 142 | 14.89 | 6980 | 68 | 18 | 1 | 13 | 0 | 202 | 44 | 5.25 | 84 | 28 | 34 |
| 16 | 42 | 15.5 | 4200 | 63 | 25 | 0 | 12 | 0 | 160 | 45 | 5.3 | 85 | 29 | 34 |

(N= Neutrophil, L= Lymphocyte, E= Eosinophil, M=Monocyte, B=Basophil, MCV=Mean Corpuscular Volume, MCH= Mean Corpuscular Hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration)

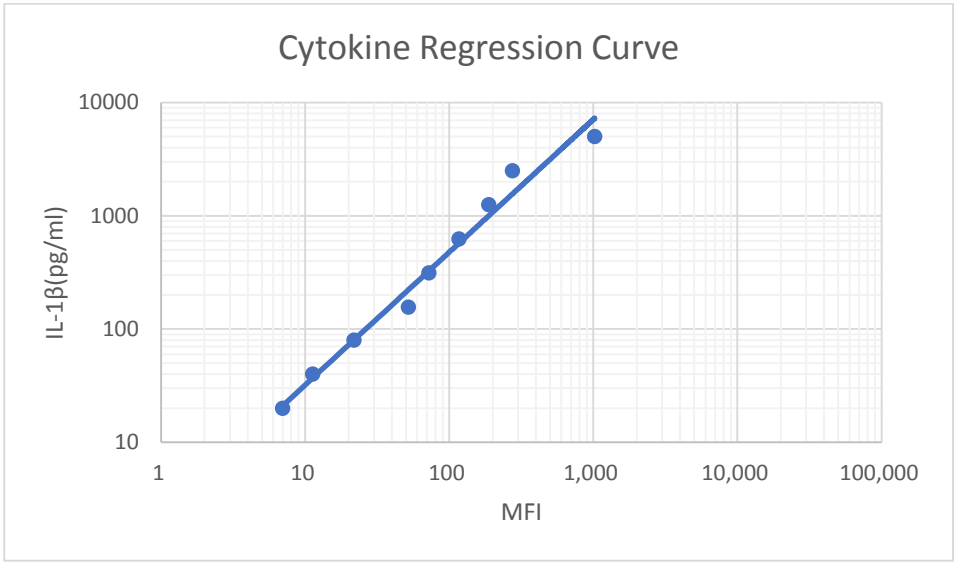
**APPENDIX 3:** Hematological Parameters of Dengue Patients During Convalescent Phase

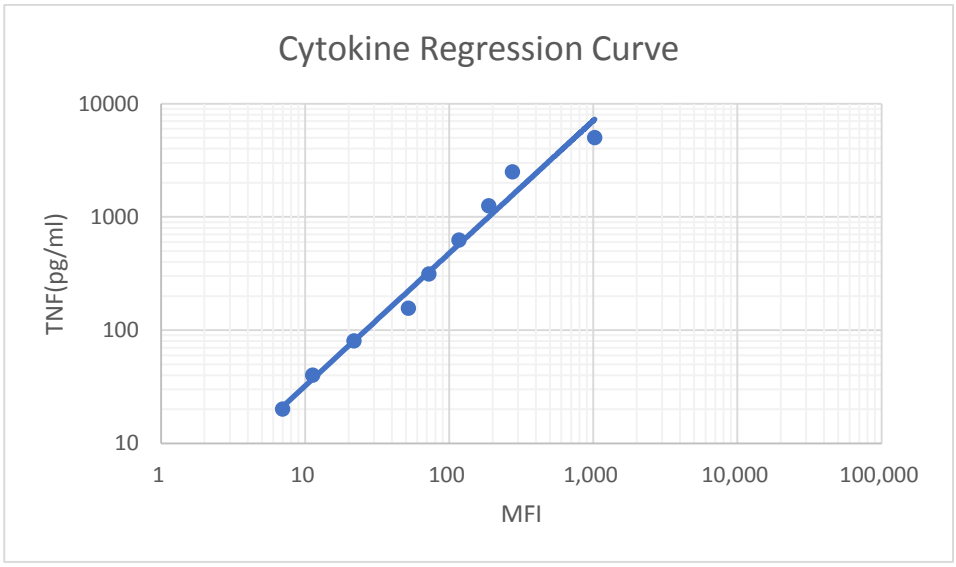
| S. N. | SAMPLE ID | Haemoglobin (Hb) (gm/dl) | Total count (TLC)/cumm | N (%) | L (%) | E (%) | M (%) | B (%) | platetes count (*1000/cumm) | PCV (%) | RBC (million/cumm) | MCV (%) | MCH (%) | MCHC (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 294 | 14.74 | 3500 | 44 | 40 | 6 | 10 | 0 | 151 | 42 | 4.78 | 89 | 31 | 35 |
| 2 | 314 | 16.6 | 8800 | 58 | 32 | 2 | 8 | 0 | 235 | 48 | 5.72 | 85 | 29 | 34 |
| 3 | 241 | 11.35 | 5780 | 46 | 46 | 1 | 7 | 0 | 310 | 32 | 2.85 | 113 | 40 | 35 |

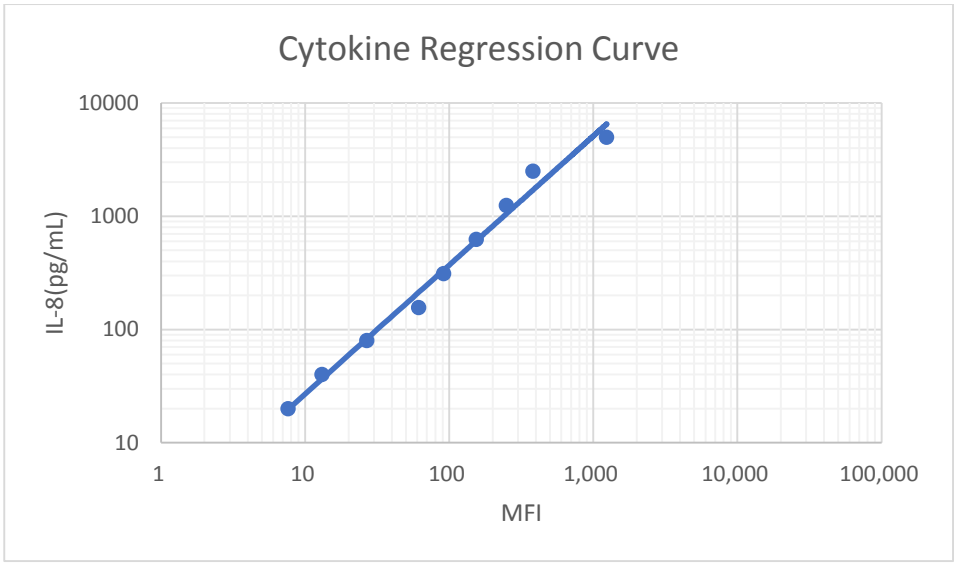
| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 4 | 282 | 15.53 | 6870 | 52 | 36 | 2 | 10 | 0 | 198 | 44 | 5.09 | 87 | 31 | 35 |
| 5 | 307 | 13.24 | 12590 | 63 | 28 | 3 | 6 | 0 | 260 | 38 | 5.07 | 75 | 26 | 35 |
| 6 | 249 | 13.54 | 7590 | 53 | 35 | 4 | 8 | 0 | 263 | 41 | 6.4 | 65 | 21 | 33 |
| 7 | 97 | 15.21 | 7680 | 62 | 28 | 2 | 8 | 0 | 209 | 45 | 5.65 | 80 | 27 | 34 |
| 8 | 242 | 11.77 | 6030 | 53 | 32 | 4 | 11 | 0 | 369 | 34 | 3.89 | 88 | 30 | 34 |
| 9 | 245 | 14.51 | 10430 | 66 | 17 | 8 | 9 | 0 | 246 | 41 | 4.29 | 95 | 34 | 36 |
| 10 | 160 | 13.31 | 5300 | 65 | 24 | 1 | 10 | 0 | 683 | 40 | 4.44 | 89 | 30 | 34 |
| 11 | 64 | 13.29 | 9420 | 61 | 27 | 4 | 8 | 0 | 243 | 39 | 4.49 | 87 | 30 | 34 |
| 12 | 119 | 15.49 | 5540 | 48 | 40 | 2 | 10 | 0 | 158 | 46 | 5.05 | 90 | 31 | 34 |
| 13 | 69 | 16.64 | 7120 | 50 | 35 | 5 | 10 | 0 | 242 | 48 | 5.47 | 88 | 30 | 35 |
| 14 | 65 | 15.87 | 8420 | 41 | 44 | 6 | 9 | 0 | 211 | 46 | 4.91 | 93 | 32 | 35 |
| 15 | 142 | 14.96 | 6690 | 54 | 37 | 1 | 8 | 0 | 200 | 42 | 5.07 | 84 | 30 | 35 |
| 16 | 42 | 14.5 | 9470 | 74 | 19 | 1 | 6 | 0 | 272 | 41 | 4.86 | 85 | 30 | 35 |

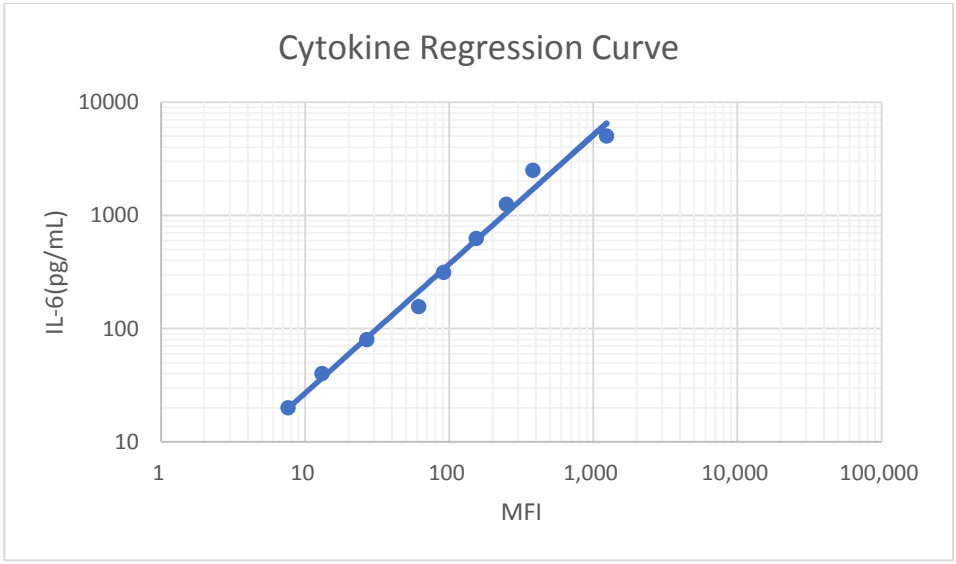
(N= Neutrophil, L= Lymphocyte, E= Eosinophil, M=Monocyte, B=Basophil, MCV=Mean Corpuscular Volume, MCH= Mean Corpuscular Hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration)

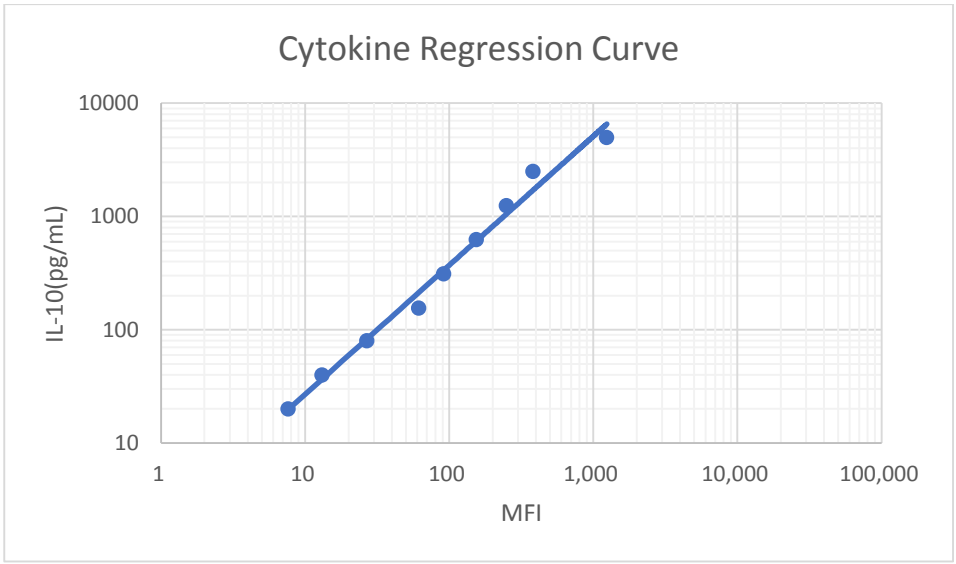
**APPENDIX 4:** Calibration Curve used to calculate the concentration of cytokines from mean fluorescence intensity obtained from flow cytometry




Cytokine Regression Curve
10000
1000
100
10
IL-8(pg/mL)
1
10
100
1,000
10,000
100,000
MFI


Cytokine Regression Curve
10000
1000
100
10
IL-6(pg/mL)
1
10
100
1,000
10,000
100,000
MFI


Cytokine Regression Curve
10000
1000
100
10
IL-10(pg/mL)
1
10
100
1,000
10,000
100,000
MFI

**APPENDIX 5:** Concentration (in pg/ml) of different cytokines in the dengue samples during acute phase

| S.N. | Sample ID | IL-8 | IL-1β | IL-6 | IL-10 | TNF | IL-12p70 |
|---|---|---|---|---|---|---|---|
| 1 | D-160 | 283.93 | 25.46 | 58.53 | 20.81 | 18.83 | 15.67 |
| 2 | D-64 | 70.21 | 21.65 | 46.03 | 35.88 | 15.37 | 15.08 |
| 3 | D-97 | 33.45 | 21.94 | 55.93 | 17.34 | 15.95 | 159.45 |
| 4 | D-69 | 78.22 | 21.01 | 26.74 | 16.53 | 15.63 | 15.62 |
| 5 | D-65 | 31.44 | 21.42 | 32.09 | 38.96 | 15.9 | 15.67 |
| 6 | D142 | 34.26 | 21.09 | 42.06 | 25.05 | 16.11 | 16.32 |
| 7 | D-42 | 393.73 | 20.57 | 35.17 | 16.21 | 15.58 | 14.92 |
| 8 | D-123 | 42.99 | 21.24 | 33.63 | 27.64 | 15.58 | 15.4 |
| 9 | D_242 | 23.42 | 13.81 | 202.44 | 95.62 | 12.93 | 18.35 |
| 10 | D_241 | 29.39 | 12.95 | 25.96 | 20.52 | 14.13 | 15.51 |
| 11 | D_245 | 34.2 | 13.66 | 26.25 | 15.43 | 13.14 | 15.33 |
| 12 | D_282 | 32.9 | 14.29 | 18.35 | 20.33 | 17.85 | 14.7 |
| 13 | D_294 | 154.48 | 14.24 | 25.09 | 28.14 | 14.84 | 17.24 |
| 14 | D_249 | 39.24 | 14.09 | 20.87 | 18.98 | 30.12 | 19.19 |
| 15 | D_307 | 24.95 | 13.76 | 19.53 | 19.3 | 18.72 | 15.33 |
| 16 | D_314 | 24.76 | 30 | 101.46 | 30.72 | 52.42 | 35.52 |

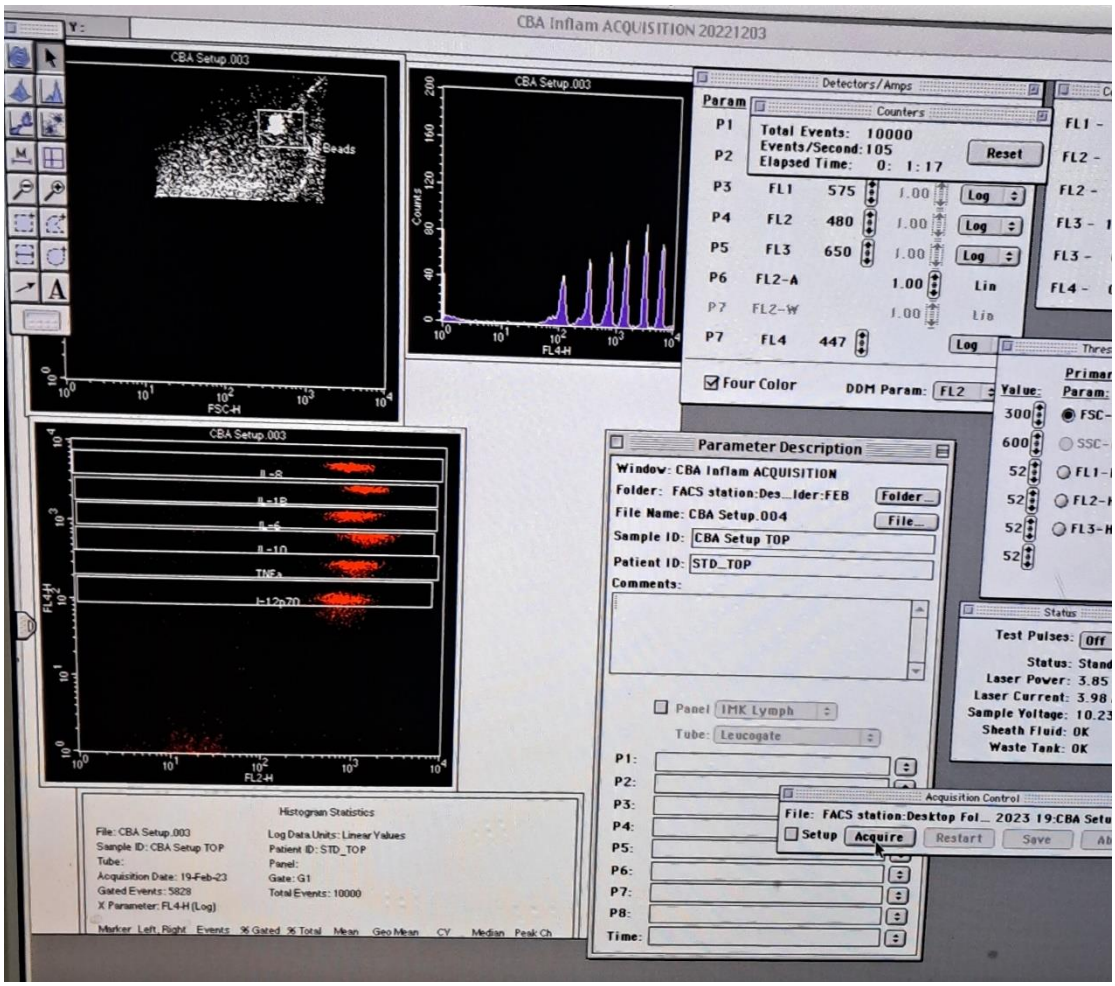
**APPENDIX 6:** Concentration (in pg/ml) of different cytokines in the dengue samples during convalescent phase

| S.N. | Sample ID | IL-8 | IL-1β | t IL-6 | IL-10 | TNF | IL-12p70 |
|---|---|---|---|---|---|---|---|
| 1 | D-160 | 40.04 | 19.61 | 79.7 | 17.66 | 211.03 | 15.73 |
| 2 | D-64 | 37.96 | 20.31 | 30.9 | 17.26 | 15.31 | 15.4 |
| 3 | D-97 | 29.02 | 20.64 | 18.44 | 14.11 | 14.78 | 188.82 |
| 4 | D-69 | 33.45 | 18.03 | 17.03 | 14.52 | 14.36 | 14.97 |
| 5 | D-65 | 25.94 | 27.7 | 15.54 | 13.95 | 16.48 | 15.35 |
| 6 | D142 | 26.83 | 21.98 | 22 | 16.13 | 16 | 16.65 |
| 7 | D-42 | 34.26 | 21.79 | 18.49 | 17.09 | 25.13 | 17.68 |
| 8 | D-123 | 30.64 | 17.45 | 15.54 | 15.64 | 14.26 | 14.06 |
| 9 | D_242 | 24.05 | 12.81 | 18.35 | 14.24 | 13.49 | 14.7 |
| 10 | D_241 | 30.19 | 12.1 | 16.76 | 15.18 | 13.14 | 14.25 |
| 11 | D_245 | 20.43 | 12.9 | 16.29 | 14.37 | 13.7 | 14.07 |
| 12 | D_282 | 21.54 | 12.43 | 13.86 | 13.75 | 13.35 | 19.29 |
| 13 | D_294 | 22.35 | 12 | 16.01 | 15.31 | 13.49 | 16.05 |
| 14 | D_249 | 18.49 | 12.71 | 14.19 | 12.94 | 13 | 28.58 |
| 15 | D_307 | 21.54 | 12.52 | 13.86 | 13.81 | 12.86 | 14.79 |
| 16 | D_314 | 20.74 | 12.48 | 27.94 | 13.5 | 12.93 | 13.9 |

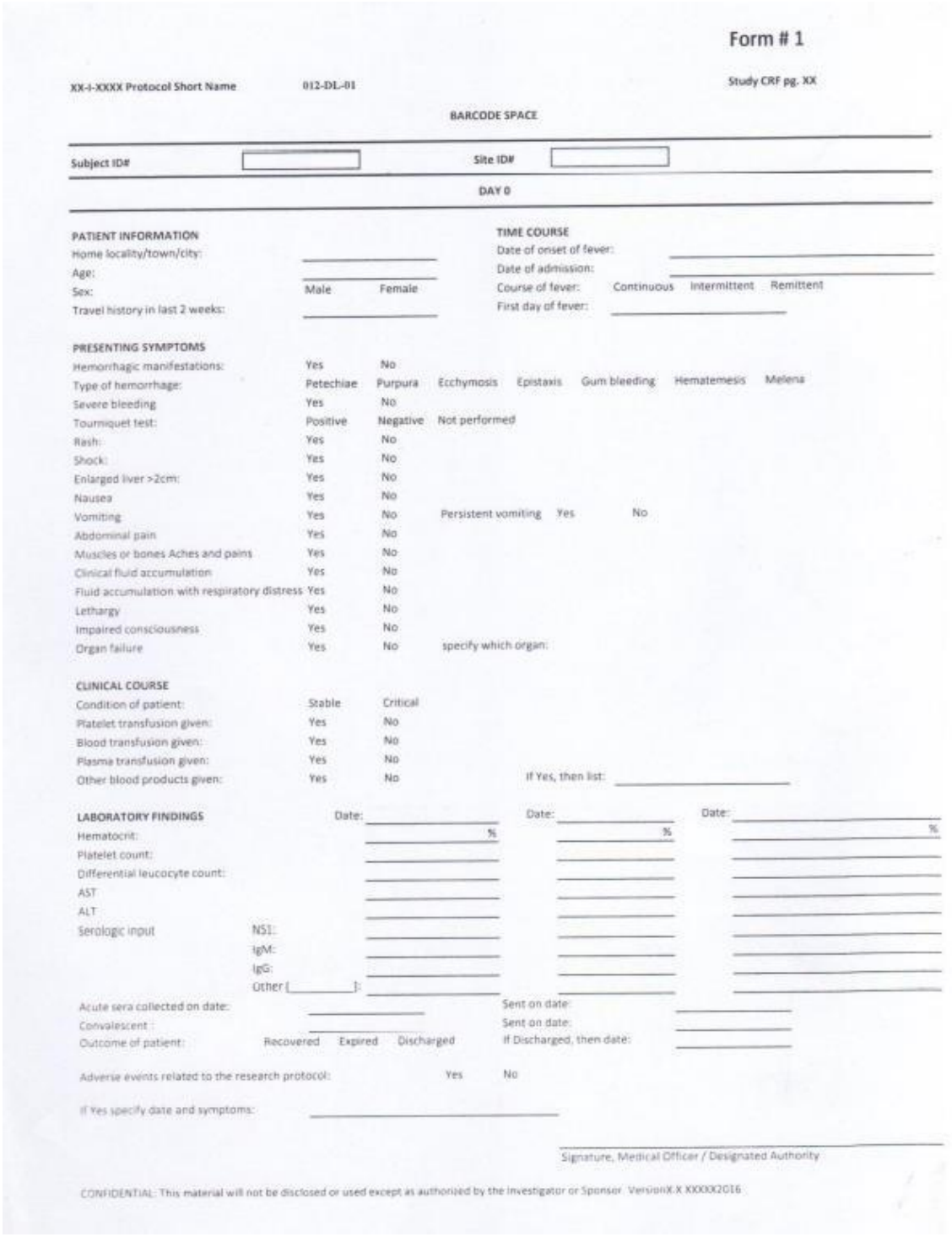
**APPENDIX 7:** Concentration (in pg/ml) of different cytokines in the healthy controls

| S.N. | Sample ID | IL-6 | IL-1β | IL-8 | IL-12p 70 | IL-10 | TNF |
|---|---|---|---|---|---|---|---|
| 1 | H2 | 14.86 | 13.47 | 24.56 | 14.97 | 14.43 | 13.84 |
| 2 | H3 | 20.31 | 16.70 | 30.73 | 17.33 | 17.71 | 18.94 |
| 3 | H4 | 20.16 | 12.85 | 46.49 | 15.15 | 14.87 | 13.91 |
| 4 | H5 | 20.73 | 18.32 | 26.69 | 14.43 | 15.50 | 17.42 |
| 5 | H6 | 15.33 | 12.00 | 47.97 | 14.79 | 14.62 | 13.21 |
| 6 | H7 | 14.59 | 16.31 | 31.47 | 14.34 | 14.31 | 21.87 |
| 7 | H8 | 16.01 | 12.67 | 25.91 | 14.52 | 30.39 | 13.28 |
| 8 | H9 | 20.02 | 13.14 | 40.38 | 19.57 | 17.07 | 13.42 |
| 9 | H11 | 15.60 | 14.72 | 21.36 | 15.15 | 16.06 | 22.91 |
| 10 | H12 | 16.08 | 16.80 | 36.42 | 15.24 | 13.68 | 13.56 |
| 11 | H13 | 15.06 | 13.19 | 48.79 | 14.79 | 14.24 | 13.35 |
| 12 | H15 | 13.46 | 12.29 | 42.25 | 14.25 | 15.00 | 13.14 |
| 13 | H1 | 18.28 | 16.51 | 25.33 | 17.61 | 15.56 | 18.07 |

**APPENDIX 8:** Acquisition of sample in the flow cytometry



**APPENDIX 9:** CRF form



Form # 1

XX-I-XXXX Protocol Short Name 012-DL-01 Study CRF pg. XX

BARCODE SPACE

Subject ID# Site ID#

DAY 0

**PATIENT INFORMATION**

Home locality/town/city: _____

Age: _____

Sex: Male Female

Travel history in last 2 weeks: _____

**TIME COURSE**

Date of onset of fever: _____

Date of admission: _____

Course of fever: Continuous Intermittent Remittent

First day of fever: _____

**PRESENTING SYMPTOMS**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Hemorrhagic manifestations: | Yes | No | | | | | |
| Type of hemorrhage: | Petechiae | Purpura | Ecchymosis | Epistaxis | Gum bleeding | Hematemesis | Melena |
| Severe bleeding | Yes | No | | | | | |
| Tourniquet test: | Positive | Negative | Not performed | | | | |
| Rash: | Yes | No | | | | | |
| Shock: | Yes | No | | | | | |
| Enlarged liver >2cm: | Yes | No | | | | | |
| Nausea | Yes | No | | | | | |
| Vomiting | Yes | No | Persistent vomiting | Yes | No | | |
| Abdominal pain | Yes | No | | | | | |
| Muscles or bones Aches and pains | Yes | No | | | | | |
| Clinical fluid accumulation | Yes | No | | | | | |
| Fluid accumulation with respiratory distress | Yes | No | | | | | |
| Lethargy | Yes | No | | | | | |
| Impaired consciousness | Yes | No | | | | | |
| Organ failure | Yes | No | specify which organ: | | | | |

**CLINICAL COURSE**

| | | |
|---|---|---|
| Condition of patient: | Stable | Critical |
| Platelet transfusion given: | Yes | No |
| Blood transfusion given: | Yes | No |
| Plasma transfusion given: | Yes | No |
| Other blood products given: | Yes | No |

If Yes, then list: _____

**LABORATORY FINDINGS**

| | | Date: | Date: | Date: |
|---|---|---|---|---|
| Hematocrit: | | % | % | % |
| Platelet count: | | | | |
| Differential leucocyte count: | | | | |
| AST | | | | |
| ALT | | | | |
| Serologic input | NS1: | | | |
| | IgM: | | | |
| | IgG: | | | |
| | Other [ ]: | | | |

Acute sera collected on date: _____ Sent on date: _____

Convalescent : _____ Sent on date: _____

Outcome of patient: Recovered Expired Discharged If Discharged, then date: _____

Adverse events related to the research protocol: Yes No

If Yes specify date and symptoms: _____

_____

Signature, Medical Officer / Designated Authority

CONFIDENTIAL: This material will not be disclosed or used except as authorized by the Investigator or Sponsor VersionX.X XXXXX2016

**APPENDIX 10:** Ethical Approval from Nepal Health Research Council

Government of Nepal

# Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 2911

**8 May 2022**

**Prof. Krishna Das Manandhar**
Principal Investigator
Central Department of Biotechnology
Kathmandu

**Ref: Approval of research proposal**

**Dear Prof. Manandhar,**

This is to certify that the following protocol and related documents have been reviewed and granted approval through the expedite review process by the Expedited Review Sub-Committee meeting for it's implementation.

| | | | | |
|---|---|---|---|---|
| **Protocol Registration No/ Submitted Date** | 686/2021 P<br>27 November 2021 | | **Sponsor Protocol No** | NA |
| **Principal Investigator/s** | Prof. Krishna Das Manandhar | | **Sponsor Institution** | La Jolla Institute for Immunology San Diego, California USA |
| **Title** | Flavivirus and Coronavirus Infections in Nepal: Host Immune Response, Viral Phylogenetic, Virus and Vector Distribution and Cross-reactivity | | | |
| **Protocol Version No** | NA | | **Version Date** | NA |
| **Other Documents** | 1. Data collection tools<br>2. Informed consent form<br>3. Support letter<br>4. Assent form<br>5. MTA<br>6. Donor agreement letter | | **Risk Category** | Minimal risk |
| **Co-Investigator/s** | 1. Dr. Sujan Shrestha<br>2. Dr. Annie Elong Ngono | | | |
| **Study Site** | STIDH and Chitwan Medical College | | | |
| **Type of Review** | √ | **Expedited** | **Timeline of Study**<br>8 May 2022 to 15 January 2026 | **Frequency of continuing review**<br>Every one year |
| | ☐ | Full Board | | |

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

Government of Nepal

# Nepal Health Research Council (NHRC)

Estd. 1991

Ref. No.: 2911

| | | | |
|---|---|---|---|
| | **Meeting Date:** 6 May 2022 | **Duration of Approval**<br>8 May 2022 8 May 2023<br>**This approval will be valid one year** | |
| **Total budget of research** | **S 20,710.00** | | |
| **Ethical review processing fee** | **S 621.3** | | |

**Investigator Responsibilities**

- Any amendments shall be approved from the ERB before implementing them
- Submit progress report every 3 months
- Submit final report after completion of protocol procedures at the study site
- Report protocol deviation / violation within 7 days
- Comply with all relevant international and NHRC guidelines
- Abide by the principles of Good Clinical Practice and ethical conduct of the research

If you have any questions, please contact the Ethical Review M & E Section at NHRC.

**Thanking you,**

**Dr. Pradip Gyanwali**
**Member Secretary**

Tel: +977 1 4254220, Fax: +977 1 4262469, Ramshah Path, PO Box: 7626, Kathmandu, Nepal
Website: http://www.nhrc.gov.np, E-mail: nhrc@nhrc.gov.np

**APPENDIX 11:** Patient's Consent Form

## अनुसन्धान अध्ययनमा सहभागिता जनाउने मञ्जुरीनामा

**परियोजना संयोजक,**
प्राध्यापक कृष्णदास मानन्धर
जैविक प्रविधि केन्द्रीय विभाग
त्रिभुवन विश्वविद्यालय
किर्तिपुर, काठमाडौं, नेपाल

**परियोजना शिर्षक: नेपालमा पाईने डेङ्गु तथा डेङ्गु जस्ता बिमारीहरुको अध्ययन**

तपाँई र/वा तपाँईको बालकलाई त्रिभुवन विश्वविद्यालय जैविक प्रविधि केन्द्रीय विभाग-नेपाल, अमेरिकाको La Jolla Institute for Allergy and Immunology र KariusDx Inc. बीचको संयुक्त प्रयासमा नेपालमा फैलिएको डेङ्गु जरोबारे वृस्तीत अध्ययन गर्न संचालन भएको "नेपालमा पाईने डेङ्गु तथा डेङ्गु जस्ता बिमारीहरुको अध्ययन" विषयको परियोजनामा सहभागि हुन आमन्त्रण गरेका छौं । यस अध्ययनबाट प्राप्त जानकारीहरुले डेङ्गु तथा डेङ्गु जस्ता बिमारीहरुबारे र तपाँई/तपाँईको बालकको राम्रो उपचार, त्यस्ता रोगहरुको खोपको विकाश र रोग पता लगाउने किट/उपकरण/बनाउनमा सहयोग पुऱ्याउनेछ । तपाँई/तपाँईको बालकलाई .....अस्पतालको जाँचमा डेङ्गु तथा डेङ्गु जस्तै रोगको लक्षण देखा परेकोले यस परियोजनामा सहभागिताको लागि छानिएको छ । यस परियोजनामा सामेल हुने वा नहुने तपाँई/तपाँईको बालकको इच्छा हो र रोजाईको कारणले यहांले पाउने उपचारमा कुनै किसिमको असर पर्ने छैन । यस अध्ययनमा सहभागिता जनाए पनि परियोजनाको कुनै पनि समयमा तपाँई/तपाँईको बालक यस अनुसन्धानबाट हट्न सक्नु हुनेछ । यस अध्ययनमा दुई भाग छन् जुन तपाँईलाई भन्नेछौं र तपाँई/तपाँईको बालकले एक वा दुवैमा सहभागिता जनाउन सक्नुहुनेछ ।

### भाग १: नमुना संकलन तथा भविष्यमा अनुसन्धान गर्न भण्डारणको लागि मञ्जुरीनामा

यदी यस अनुसन्धानमा सामेल हुन चाहनु भएमा हामी तपाँई/तपाँईको बालकको स्वास्थ्य, जस्तै पुराना स्वास्थ्य सम्बन्धि समस्याहरु र जाँचबाट पत्ता लगाएका उपायहरु र प्रयोगशालाका नियमित परिक्षणका विवरण सम्बन्धि कुराहरु बुझ्नेछौं । यदी तपाँईले सामेल हुने निर्णय गरिसक्नु भएको भए अस्पतालमा नियमित परिक्षणको समयमा तपाँई/तपाँईको बालकको २ पटकको रगतको नमूना संकलन गरिनेछ । बच्चाको भए करिब १ चिया चम्चा (५ मि.लि.) र वयस्क व्यक्ति भए ३ चिया चम्चा (१५ मि.लि.) रगत लिईनेछ । पहिलो रगतको नमूना तपाँई पहिलो पटक अस्पतालमा जाँच गराउन आउँदा र दोस्रो पटक ४ हप्ता पछी डेङ्गु तथा डेङ्गु जस्तै रोग बाँकी छ कि छैन भनी जाँच गराउन आउँदा लिईनेछ । यदी अध्ययनमा समावेस हुनु भएकोछ भने तपाँई/तपाँईको बालकलाई दोस्रो पटक रगतको नमूना लिन बोलाईनेछ । यसमा सहभागि भए बापट कुनै पैसा लाग्ने छैन ।

संकलित रगतको नमूनाको जाँचबाट डेङ्गु तथा डेङ्गु जस्तै रोगसग तपाँई/तपाँईको बालकको शरिरले गरेको प्रतिक्रिया र भाईरस बारे थप जानकारी प्राप्त गर्न सजिलो हुनेछ । साथ साथै रगत परिक्षणबाट अरु राम्रो डेङ्गु रोग पत्ता लगाउन सहयोग मिल्नेछ । अनुसन्धानका क्रममा केही रगत परिक्षण त्रिभुवन विश्व विद्यालय जैविक प्रविधि केन्द्रीय विभाग, नेपालमा हुन्छ भने केही नमूना La Jolla Institute for

Allergy and Immunology तथा KariusDx Inc. मा गर्न अमेरिकामा पठाईनेछ । KariusDx Inc.मा १८ वर्ष र माथिका व्यक्तिहरुको रगतको नमूना मात्र परिक्षणको लागि पठाईनेछ ।

तपाँई/तपाँईको बालकको अस्पतालमा जाँच भएको रगतको विवरण डाक्टरले उपलब्ध गराउनेछ तर अनुसन्धानको विवरण भने तपाँईलाई दिईने छैन तथापि यसबाट तपाँईको उपचारमा कुनै पनि बाधा पर्ने छैन ।

तपाँई/तपाँईको बालकलाई दुई बटा कुरामा एक बटा छान्न अनुरोध गरिनेछ, क) तपाँई/तपाँईको बालकको रगतको नमुना भविष्यमा अध्ययनको लागि पनि राख्ने वा ख) तपाँई/तपाँईको बालकको रगतको नमुना यस परियोजना पछी नष्ट गर्ने । यदी तपाँई आफ्नो नमूना भविष्यमा गरिने परिक्षणको लागि भण्डारण गर्न रोज्नु भएको छ भने यस परियोजना पछीको बाँकी नमूना र विवरणलाई KariusDx Inc ले अनुसन्धान तथा रगत परिक्षणबाट संक्रमित रोगहरु पत्ता लगाउने कार्यको लागि प्रयोग गर्न सक्नेछ ।

कृपया तलका कोठामा चिनो लगाउनु होस जसले तपाँई/तपाँईको बालकको रगतको नमूना भविष्यमा गरिने अनुसन्धानको लागि राख्ने/नराख्ने भन्ने रोजाई देखाउँछ ।

☐ म/मेरो बालकको स्वास्थ्य विवरण र बाँकी रगतको नमुना भविष्यमा गरिने अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छ ।**

☐ म/मेरो बालकको स्वास्थ्य विवरण र बाँकी रगतको नमुना भविष्यमा गरिने अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छैन ।।**

**भाग २: वंशानुगत अध्ययनको लागि थप मञ्जुरी**

हामी, तपाँई/तपाँईको बालकको रगत नमूना भविष्यमा गरिने वंशानुगत अनुसन्धानको लागि पनि राख्न मञ्जुरी चाहन्छौं । वंशानुगत अनुसन्धान जिन (Gene)को अध्ययन हो । Genes मा डि.एन.ए. (DNA) र आर.एन.ए. (RNA) हुन्छ । DNA ले हाम्रो शरिरले कसरी काम गर्ने र रोग तथा बाहिरी वातावरणलाई प्रतिक्रिया दिने कामको आदेश दिन्छ भने RNA ले वाहकको काम गर्छ जसले आदेश र वंशानुगत विवरणहरु शरिरको आवश्यक भागहरुमा पुऱ्याउँछ । Genes वंशानुगत भएकोले आमा बुबाबाट बच्चामा आएको हुन्छ । यस अनुसन्धानमा ती Genes हरुको अध्ययन गरिन्छ जसले डेङ्गु तथा डेङ्गु जस्ता रोगहरु अधिकतम रुपमा ग्रसित गर्न जिम्मेवार छन् साथ साथै स्वास्थ्यसंग सम्बन्धित अन्य Genes को पनि अध्ययन गर्नेछ । यो अनुसन्धान गर्न धेरै वर्ष लाग्ने अनुमान गरिएकोछ । गोप्यताको बारेमा भाग १ मा उल्लेख गरिए जस्ते व्यवस्थित गरिनेछ । यदी तपाँईलाई यस अनुसन्धानमा सहभागिता जनाउन मन नलागेमा माथि उल्लेखित भाग १ को लागि मात्र सहभागिता जनाउन सक्नु हुनेछ र यस रोजाईले तपाँई/तपाँईको बालकको स्वास्थ्य उपचारमा कुनै बाधा पर्ने छैन ।

यदी यस जाँच तथा अनुसन्धान (भाग २)मा तपाँई/तपाँईको बालक संलग्न हुने निर्णय गर्नु भएको छ भने रगतको नमूनाहरुमा तपाँई/तपाँईको बालकको नाम र ठेगाना नखुल्ने गरी सांकेतिक अंक राखिईनेछ र नेपालको त्रिभुवन विश्वविद्यालय, अमेरिकाको La Jolla Institute for Allergy and Immunology तथा KariusDx Inc. मा पठाईनेछ । तपाँई/तपाँईको बालकको पहिचान परियोजनाका अनुसन्धानकर्तालाई हुनेछैन । अर्को शब्दमा तपाँई/तपाँईको बालकको नाम अनभिज्ञ रहनेछ ।

तपाईको नमूनाहरु र विवरणहरु अनुसन्धानको लागि प्रयोग हुनेछ र हुन सक्छ यसबाट नौलो चीज, रोग जाँच र औषधि उपचारका नयाँ प्रविधि पत्ता लाग्न सक्छ । तपाँई/तपाँईको बालकलाई यस अनुसन्धानबाट कुनै किसिमको सिधै वा नगदी फाईदा भने हुने छैन तर भविष्यका सन्ततीलाई यसबाट आर्जित ज्ञान फाइदाजनक हुनसक्छ । तपाँई/तपाँईको बालकलाई यस वंशानुगत अध्ययनको परिणाम बारे जानकारी गराइनेछैन र तपाँई/तपाँईको बालकको उपचारमा उपयोग गरिनेछैन ।

यदी पछी पनि तपाँईलाई तपाँई/तपाँईको बालकको रगत नमूना र विवरण भविष्यमा गरिने वंशानुगत अनुसन्धानको लागि राख्न चाहनु भएन भने कृपया हामीलाई खबर गर्नु होला हामी तपाँई/तपाँईको बालकको रगतबाट थप अध्ययन नगर्न भरसक प्रयास गर्नेछौं ।

कृपया तलका कोठामा चिनो लगाउनु होस जसले तपाँई/तपाँईको बालकको DNA/RNA को नमूना भविष्यमा गरिने वंशानुगत अनुसन्धानको लागि राख्ने/नराख्ने भन्ने इच्छा देखाउँछ ।

☐ म/मेरो बालकको स्वास्थ्य विवरण र DNA/RNA भविष्यमा गरिने वंशानुगत अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छ** ।

☐ म/मेरो बालकको स्वास्थ्य विवरण र DNA/RNA भविष्यमा गरिने वंशानुगत अनुसन्धानको लागि पनि राख्न **मेरो मञ्जुरी छैन** ।।

यदी यस अध्ययनमा तपाँई/तपाँईको बालक संलग्न हुने निर्णय गर्नु भएको छ भने यसमा धेरै खटरा र फाईदा छन् । सम्भाव्य खटरामा रगत निकाल्दा सियो घोचेको स्थानमा क्षणिक सुनिनु र चिलाउनु र कहिले काहीं घाउ बन्नु हुन् । ती सम्भाव्य खटराहरुलाई कम गर्न निपुण नर्स/दक्ष स्वास्थ्य कर्मिद्वारा स्टेराईल सियो प्रयोग गरी रगत निकालिनेछ ।

अनपेक्षित घटना भई तपाँई/तपाँईको बालकलाई रगत दिँदै वा दिएको कारणबाट चोट लाग्न गएमा अस्पतालमा उपचारको व्यवस्था गरिनेछ । उपचार बापट लाग्ने खर्च La Jolla Institute for Allergy and Immunology, अमेरिकाले व्यहोर्नेछ । तर तपाँईलाई विरामी अवधिभर कुनै किसिमको खर्च दिइनेछैन । यस सम्बन्धि अन्य कुनै प्रश्न छ भने अस्पतालको तल उल्लेखित डाक्टरलाई सम्पर्क राख्नु होस ।

यस अध्ययनबाट प्राप्त तपाँई/तपाँईको बालकको स्वास्थ्यका विवरणहरु गोप्य राखिनेछ र तपाँई/तपाँईको बालकको नाम र पहिचान अध्ययनको कुनै पनि प्रतिवेदनमा उल्लेख गरिने छैन । सबै विवरणहरु र नमूनाहरु सांकेतिक अंक राखेर प्रयोग तथा भण्डारण गरिनेछन । सो सांकेतिक अंकको पहिचान परियोजनाका संयोजक र प्रमुख व्यक्तिहरुलाई मात्र थाहा हुनेछ । तपाँई/तपाँईको बालकको पहिचान बाहिर आउने सम्भावना असम्भव जस्तै छ तथापि हामी सम्भावनाहरु न्यून गर्न लागि परेका हुनेछौं ।

यदी तपाँईसंग यस अध्ययन सम्बन्धि कुनै प्रश्न, जिज्ञासा तथा समस्या छ भने त्रि.वि. जैविक प्रविधि केन्द्रीय विभागका प्रा.डा. कृष्णदास मानन्धरलाई फोन नं. १ ४३३६२२१ मा सम्पर्क राख्न सक्नु हुनेछ । यदी तपाँईलाई यसमा संलग्न भए बापट आफ्नो अधिकार सम्बन्धि प्रश्न छ भने यस अस्पतालका डाक्टर श्री ..... लाई अस्पतालको फोन नं/ईमेल ..... मा सम्पर्क राखी सोध्न सक्नु हुनेछ । यदी तपाँईलाई यस अनुसन्धानमा सहभागिता जनाउन मन नलागेमा माथि उल्लेखित डाक्टर वा सेवामा रहेकी नर्सलाई सम्पर्क राखी जानकारी दिन सक्नु हुनेछ ।

यस अध्ययनबाट प्राप्त ज्ञानले डाक्टरलाई नेपाल र संसारभरका बालक तथा वयस्क व्यक्तिहरुको डेङ्गुको उपचार गर्न महत पुग्नेछ, रोग परिक्षण गर्ने नयाँ विधिको परिक्षण गर्न सकिनेछ र यस रोगको भ्याक्सीन बनाउन वैज्ञानिक आधारहरु तयार गर्न सहयोगि हुनेछ । फेरि पनि एक पटक भन्न चाहन्छु कि यस अध्ययनमा संलग्न हुनु भनेको स्वयंसेवकको काम गर्नु हो र तपाँई कुनै पनि बेला यसबाट अलग हुन सक्नु हुनेछ ।

**तपाँईसंग कुनै प्रश्न छ ?**

यस मञ्जुरी पत्रमा लेखिएका सबै कुराहरु मलाई पढेर सुनाईयो । मेरो प्रश्नहरुको चित्त बुझ्दो जवाफ पाएं र मैले थाहा पाएं कि म/मेरो बालकको यस अध्ययन अनुसन्धानमा सहभागिता स्वयंसेवकको रुपमा हो र म/मेरो बालकको स्वास्थ्य उपचारमा कुनै असर नपर्ने गरी सहभागित जनाउन पनि सक्छु र कुनै पनि समयमा नाम फिर्ता पनि लिन सक्छु ।

_____ _____

सहभागिको नाम अध्ययनको सांकेटिक अंक

_____

बाबु आमा वा अभिभावकको नाम (आवश्यक भए)

_____ _____

बाबु आमा वा अभिभावकको दस्तखत मिति

**सहभागि बच्चालाई पनि बुझाउने काम _____ पुरा भयो वा _____ पुरा गरिएको छैन ।**

**यदी पढ्न र लेख्न असक्षम छ भने:**

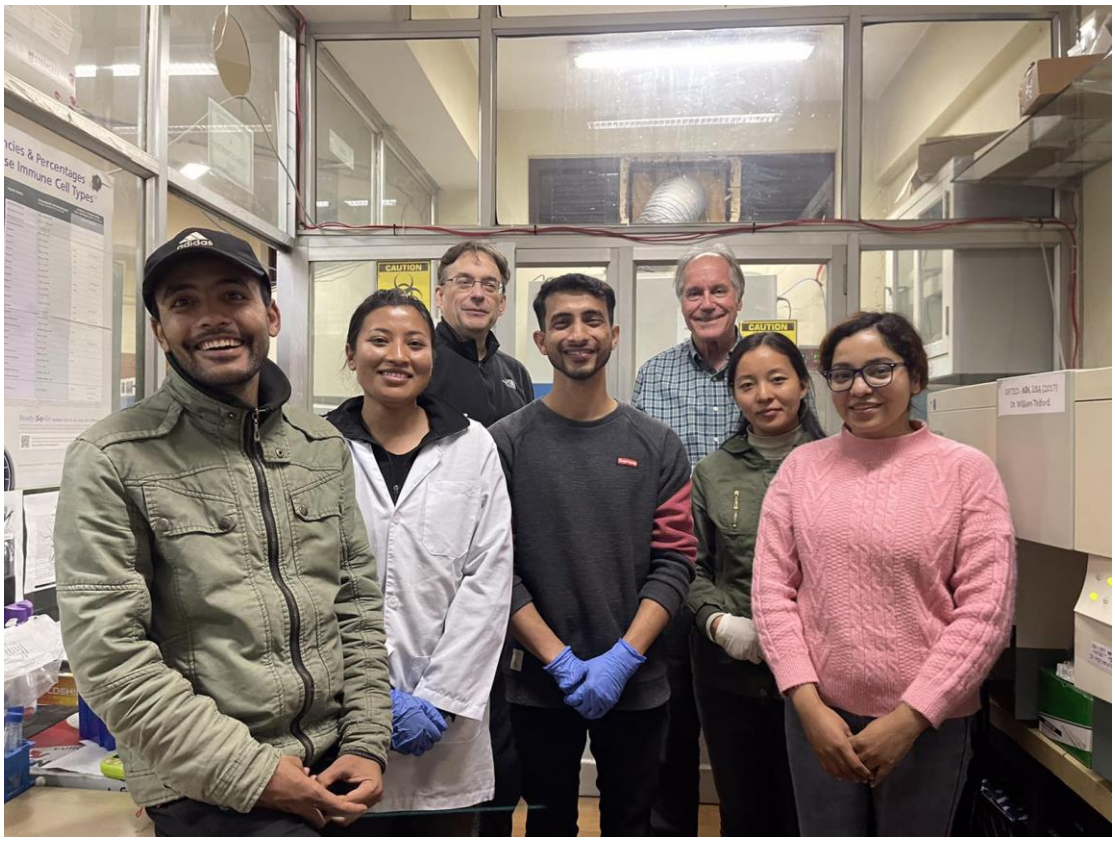
म यस मञ्जुरीनामाको साक्षीले यसमा उल्लेखित कुराहरु ठिक संग पढेर संभाव्य सहभागि व्यक्तिलाई सुनाएं र नीजले यसबारे प्रश्न गर्ने मौका पाए । म आश्वस्त पार्छु कि नीजले मञ्जुरी आफ्नो इच्छा बमोजिम दिएका हुन् ।

_____

साक्षीको नाम

_____ _____

**APPENDIX 12:** Photos and Memories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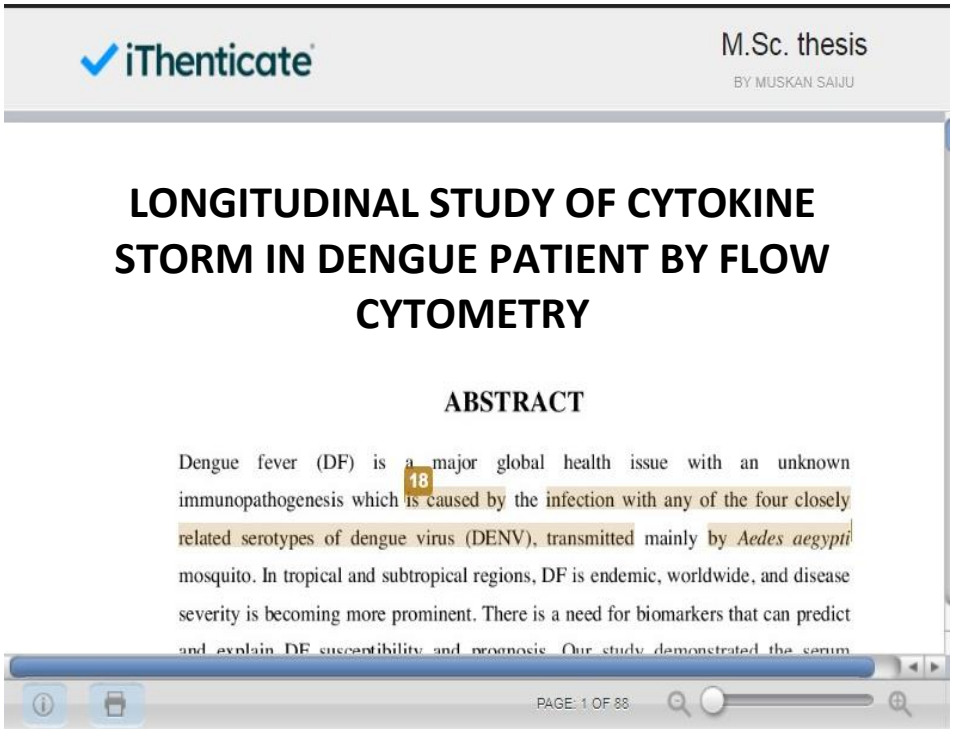
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BY MUSKAN SAIJU
LONGITUDINAL STUDY OF CYTOKINE STORM IN DENGUE PATIENT BY FLOW CYTOMETRY
ABSTRACT
Dengue fever (DF) is a major global health issue with an unknown immunopathogenesis which is caused by the infection with any of the four closely related serotypes of dengue virus (DENV), transmitted mainly by Aedes aegypti mosquito. In tropical and subtropical regions, DF is endemic, worldwide, and disease severity is becoming more prominent. There is a need for biomarkers that can predict
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