CHAPTER 1

INTRODUCTION

CHAPTER ONE: INTRODUCTION

1.1 Overall introduction of project

Dengue is a major vector-borne disease in tropical and sub-tropical areas of the world. Among the symptoms presented in patients with dengue infection are headache, fever, rash, pain behind the eyes, muscle and joint pains. In the case of severe dengue infections, hemorrhagic manifestations could develop. The first dengue case in Malaysia was reported in Penang back in 1902 (Skae, 1902), while the first major outbreak of dengue hemorrhagic fever (DHF) was reported in 1973 with dengue virus serotype 3 implicated as the dominant serotype (George, 1992). In 1982, a large dengue epidemic was reported with 3,005 notified cases, of which 28.4% were DHF. According to the Western Pacific Regional Office of the World Health Organization, 39,222 dengue cases were reported in Malaysia in 2013, with 83 deaths.

Currently, there are no suitable vaccines, specific treatment nor anti-virals for the treatment of dengue. Thus, patients are managed according to symptoms. In Malaysia, *Carica papaya* leaves (CPL) juice has been used as a house-hold remedy for dengue infection. Usually the leaves are blended and squeezed to produce the juice that was then consumed. Ahmad *et al.* (2011) reported that the level of platelet count, white blood cell and neutrophils increased in dengue patients after consuming CPL for five consecutive days. The exact role of CPL juice is unknown but is believed to have some immune modulation qualities (Indran, Mahmood, & Kuppusamy, 2008).

Therefore, in this study, we aim to investigate the use of CPL juice as a treatment procedure for dengue infection. In order to achieve this target, we used two-dimension gel

electrophoresis (2D-GE) as this technique is generally used for proteome profiling, to compare the expression of two or more complex protein samples, for the localization and identification of post-translational modifications, and for the study of protein–protein interactions (Chandramouli & Qian, 2009). Profiles of protein expression in serum of dengue patients before and after consuming CPL juice will be analysed and differential proteins of interest will be identified. The findings may shed some insights on the role of CPL in the immune modulation of dengue patients.

1.2 Objectives of study

The objectives of this study are:

i. To determine dengue serotype among patients recruited in the study

ii. To develop and compare 2D-GE profile of proteins in sera of dengue patients before and after treatment with CPL juice

iii. To identify differentially expressed protein from the above patients using mass spectrometry (MS) analysis and to correlate the role of the proteins with dengue pathology and immune modulation

CHAPTER 2

LITERATURE REVIEW

CHAPTER TWO: LITERATURE REVIEW

2.1 Overview of dengue

Dengue is an endemic disease that affects tropical and subtropical regions around the world, predominantly in urban and semi-urban areas. Dengue virus (DENV) belongs to the genus of Flavivirus and family *Flaviviridae* (Rivetz *et al*., 2009). The virus is transmitted to human through the bites of Aedes mosquitoes, mainly *A. aegypti* and *A. albopictus* (WHO, 1997). The female mosquito feeds during daytime, usually in the morning and late afternoon. According to Gubler (1998), the female of either type of mosquito must bite an infected human during the viraemic phase of the illness, which usually is within 4-5 days but could also last up to 12 days, especially among primary dengue infection. The extrinsic incubation time period is between 8 to 12 days. This refers to the time required starting from when a viraemic human is bitten to when the mosquito itself becomes infective. The incubation period for human is 4-6 days (Gubler, 1998). The infected mosquitoes remains capable of transmission for its entire life (Kautner, Robinson, & Kuhnle, 1997).

2.1.1 Dengue virus structure

A mature dengue virus particle is small and consists of a single-stranded ribonucleic acid (RNA) genome of about 11 kb. It is surrounded by an icosahedral nucleocapsid with an approximate diameter of 40 to 50 nm. The 5’ end of the RNA has a type 1 cap structure but lacks a poly-A tail at the 3’ end. The RNA codes for three structural and seven non-structural proteins (Perera & Kuhn, 2008). The three structural proteins form the components of the virion comprising of the core (C) protein, a membrane-associated (M)

protein and an envelope (E) protein. The non-structural proteins are involved in viral RNA replication and termed as NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Perera & Kuhn, 2008). The diagrammatic representation of the virus structure is shown in Figure 1.

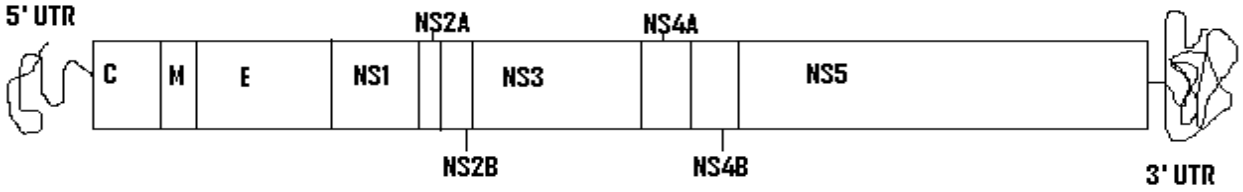

Figure 1: Dengue virus genome (adapted from Nature Publishing Group website)

The envelope of the virus is a lipid bilayer containing two envelope-associated proteins; the E and M proteins. The E protein plays an important role in blood cell hemagglutination, induction of a protective immune response and is also responsible for attachment to the cellular receptors and fusion with cell membrane. The NS1 protein is rarely found in those with primary infections but could be detected in high titers in patients with secondary infections (Kautner *et al*., 1997). NS2 is thought to be involved in polyprotein processing while NS3 codes for the viral proteinase that functions in the cytosol. NS4 is involved in the establishment of the membrane-bound RNA replication complex. NS5 is believed to be the virus-encoded RNA-dependant RNA polymerase (Kautner *et al.*, 1997).

There are four serotypes of DENV which are DENV-1, DENV-2, DENV-3 and DENV-4 (Rivetz *et al*., 2009). Infection by a given serotype induces a lifelong protective immunity against the homologous serotype, but not to others. Secondary infection with another serotype is considered to be a major risk factor for developing DHF and dengue shock syndrome (DSS) (Gubler, 1998). According to Mongkolsapaya *et al.* (2003), secondary infection by a different dengue serotype is considered the most significant risk factor for DHF/DSS as original antigenic sin in the T-cell responses may suppress or delay viral elimination, leading to higher viral loads and increased immunopathology.

Researchers found that DENV-1 and DENV-3 are more pathogenic without immune priming from other serotypes. They also reported that DENV-4 causes milder disease in primary DENV infection (Fried *et al*., 2010). Another study showed that DENV-2 and DENV-3 are associated with severe dengue disease, DHF, whereas DENV-4 is found primarily in secondary dengue infections (Nisalak *et al.*, 2003). A study in India also found out that DENV-2 and DENV-3 were causing more severe dengue infections (Gupta, Dar, Kapoor, & Broor, 2006).

2.1.2 Pathophysiology of dengue

The human body system has three lines of defense from foreign invaders that causes infection and disease. Two of them are non-specific; which do not distinguish one infectious agent from another.

2.1.2.1 The first line of defense

The first line of non-specific defense is external. It consists of epithelial tissues that cover and line our bodies and the secretions they produced. This refers to skin and mucous membranes. Skin is a barrier that cannot be penetrated by viruses.

2.1.2.2 The second line of defense

When dengue mosquito bites human, the viruses will face the second line of defense. The NS1 protein, a non-structural viral protein that is secreted from infected cells and present in blood, is known to be a major immune target (Nielsen, 2009). NS1 was identified to be up-regulated in the serum of dengue fever (DF) patients compared to healthy individual samples (Thayan *et al.*, 2009). The amount of NS1 circulating in human sera appears to be significantly higher in patients who developed DHF rather than DF (Libraty, 2002). The internal mechanism of non-specific defense in the body depends on phagocytosis, the ingestion of invading organisms by certain types of white cells. There are three types of responses involved in this defense; phagocytic white blood cell (leukocytes), the inflammatory response and innate immune system.

Neutrophils, monocytes (large version known as macrophages) and eosinophils are phagocytic cells that are involved in the phagocytosis of white blood cell. Cells that has been damaged by dengue viruses, will release chemical signals that attract neutrophils from the blood. The neutrophils will enter the infected tissue, engulf and destroy viruses there. It will self-destruct as they destroy foreign invaders and their average lifespan is only a few

days. Macrophages works by extending long pseudopodia that can attach to polysaccharides on a viruses' surface. The macrophages will engulf the virus in a vacuole that fuses with a lysosome. Lysosome will either generate toxic forms of oxygen (superoxide anion and nitric oxide, (NO)), or secrete lysosomal enzymes to digest the viral components. Eosinophils defense larger parasitic invader, such as blood fluke, by positioning themselves against the external wall of a parasite and discharge destructive enzymes from cytoplasmic granule.

The first stage in the inflammatory response following infection is recognition of the pathogen and activation of tissue macrophages. The activated macrophages produced a number of factors including prostaglandins, platelet activation factor (PAF) and cytokines. A study has shown that antibodies to NS1 antigen cross-react with platelets leading to platelet activation (Sun *et al*., 2007). Three of the cytokines produced are interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor-α (TNFα). IL-6 was reported to increase significantly in patients with severe DF (Albuquerque *et al*., 2009). Dengue virus-infected monocytes and endothelial cells were reported to produce some of multiple cytokines including TNFα (Avirutnan, Malasit, Seliger, Bhakdi, & Husmann, 1998). TNFα, PAF and the prostaglandins act directly on the endothelium to increase vascular permeability (de Jong *et al*., 2006; Kurane, 2007). The increasing in vascular permeability will results in plasma leakage. Plasma leakage will lead to elevation of hematocrit, one of the symptoms in DF. IL-1 and TNFα activate endothelial cells lining the blood vessels at the site of infection. This will dilate precapillary arterioles and constrict post capillary venules in the injured area, causing an increasing in the local blood supply. A study has shown that higher

concentration of TNFα will lead to thrombocytopenia (Martina, Koraka, & Osterhaus, 2009). PAF are involved in the releasing of histamine from basophils (a type of leukocytes) and mast cells (found in connective tissues). Histamine will trigger both dilation and increased permeability of nearby capillaries. This will promote blood flow to the site of injury. Mast cells also release heparin and proteolytic enzymes which acts the same as histamine. All of these responses that happen will aid in delivering clotting elements to the injured area. Blood clotting will help block the spread of microbes to other parts of the body of the blood vessels. Endothelial cells and monocytes will secrete chemokines to attract phagocytes to the area.

Another systemic response to infection is fever. High production of IL-1 affects the brain, causing fever, somnolence (sleepiness) and anorexia (loss of appetite). Certain leukocytes will release pyrogens, which set the body's thermostat at a higher temperature (Wood, 2006). A very high fever may be dangerous, but a moderate fever contributes to defense by inhibiting the growth of some microorganisms. Fever may also facilitate phagocytosis and, by speeding up body reactions, may speed the repair of tissues. When an individual is infected by dengue viruses, one of the symptoms that appear is high fever, ranging from 38-40 °C. This is due to the systemic response.

Excess in the production of IL-6 will effect on hepatocytes, the main tissue in the liver. This cytokine will stimulate the cells to produce a series of proteins called the acute phase proteins (APPs) (Clyde, Kyle & Harris, 2006). The level of APPs is at basal levels in

healthy individuals, but the concentration will increase following stimulation of the liver. There are two categories of positive APPs, based on the degree which they increase; 1.5- to 5- fold and 100- to 1000- fold (Wood, 2006). The proteins that increase 1.5- to 5- fold are fibrinogen (involved in clotting), haptoglobulin (involved in reducing bacteria growth), complement component C3 and mannose-binding protein (MBP). The proteins that increase 100- to 1000- fold are serum amyloid A (SAA) and C-reactive protein. SAA inhibits fever and platelet activation while the C–reactive protein helps phagocytes to recognize pathogens or damaged cells. It is also reported that SAA may suppress lymphocytic response to antigens, impair platelet aggregability, and contribute to the regulation of tissue collagenase expression. SAA induces mast cell adhesion to the extracellular matrix. This will lead to mild bleeding such as a nose bleed, bleeding gums, or easy bruising. While the levels of some proteins increases as part of the acute-phase response, the levels of albumin, transferrin, transthyretin, retinol-binding protein (RBP), antithrombin and transcortin are down-regulated, hence they are grouped as the "negative" APPs (Wood, 2006).

The last in the second line defense is the innate immune system. Among the components in this group are the interferons (IFNs) and natural killer (NK) cells. IFNs are secreted by virus-infected cells. They diffuse to neighbouring cells and induce them to produce other chemicals that inhibit viral reproduction. This will limit the spreading of virus from cell to cell. There are 3 main IFNs; IFN-α, IFN-β and IFN-γ. IFN-α and IFN-β which are produced by many cell types including macrophages, fibroblasts, lymphocytes, endothelial cells and epithelial cells. Both of these IFNs are produced when the cells are stimulated by viral products which are made only by viruses; therefore indicate viral

infection of a cell. IFN-γ is produced by lymphocytes. This IFN was found to increase significantly in patients with severe DF (Albuquerque *et al.*, 2009).

NK cells do not attach microorganisms directly but they destroy virus-infected body cells. NK cells enter sites of inflammation, where they are stimulated by cytokine IL-2, which is produced by activated macrophages, to produce IFN-γ and TNF-α (Srikiatkhachorn & Green, 2010). NK cells mount an attack on the cell's membrane, causing the cell to lyse. Elevated levels of IFN-γ and TNF-α have been reported in DHF cases (Braga *et al*., 2001)

2.1.2.3 The third line of defense

The immune system is the third line defense. There are two types of lymphocytes in a human body; B lymphocytes (B cells) and T lymphocytes (T cells). B cells are lymphocytes that continue their maturation in the bone marrow while T cells are lymphocytes that continue their maturation in the thymus. Lymphocytes are known to display specificity because it can recognize and respond to a particular microbe and other foreign molecules. A foreign (viral) particle that elicits a specific response by lymphocytes is called an antigen. An antigen elicits an immune response by activating B cells to secrete proteins called antibodies (Wood, 2006).

Each antigen has a particular molecular shape and stimulates certain B cells to secrete antibodies that interact specifically with it. This means that both lymphocytes recognize specific antigens by their plasma membrane-bound antigen receptors. However, lymphocytes do not react to most self-antigens. T cells have a crucial interaction with one important group of native molecules known as human leukocyte antigens (HLA). It is a collection of cell surface glycoproteins encoded by a family of genes. There are two main classes of HLA which are Class I HLA molecules and Class II HLA molecules. Through a process known as antigen presentation, a HLA molecule cradles a fragment of intracellular protein antigen in its hammocklike groove, carries it to cell surface and "presents" it to an antigen receptor on a nearby T cell. There are two types of T cell which responds to one of the two classes of HLA molecule; cytotoxic T cell (T_C) and Helper T cells (T_H). T_C cell has antigen receptors that bind to protein fragments displayed by the cell's Class I HLA molecules. T_H cells have receptors that bind to peptide displayed by the cell's Class II HLA molecules (Wood, 2006).

Class I HLA molecules can be found on almost all nucleated cells. These molecules will present fragments of protein made by viruses to T_C, which will then kill the virus-infected cells. In the other hand, Class II HLA molecules are restricted to a few specialized cell types, including macrophages and B cells. These molecules are also known as antigen-presenting cells (APC) which will ingest pathogens (viruses) and then destroy them. These molecules collect peptide remnants of this degradation and present them to T_H. T_H will send out chemical signals that incite other cell types to fight pathogens (Virella, 2007; Wood, 2006).

There are two types of responses to antigen; a humoral response and a cell-mediated response. In humoral response, B cell activation is involved, which will then give rise to plasma cells to secrete antibodies. When a virus enters the third defense system, a macrophage will engulf and break down the viral proteins. As a result, the small fragments of viral proteins that have been broken down will be captured by the Class II HLA molecules in its antigen-binding groove, and carries it to the surface of the macrophage, revealing the foreign peptide to a T_H. The interaction between APC and T_H is enhanced by the presence of T cell surface protein called CD4. This protein will bind to part of the class II HLA protein and helps to keep the T_H and the APC joined while activation of T_H cell occurs. The T_H cell will proliferate and differentiate into a clone of activated T_H cell and memory T_H cells. At the same time, as macrophage phygocytoses and presents antigen, the macrophage is stimulated to secrete IL-1, which will work together with the antigen to activate T_H cells to produce IL-2 and other cytokines that will stimulate other lymphocytes. For example, IL-2 helps B cells that have interact with the antigen, to differentiate into antibody-secreting plasma cells. IL-2 also helps T_C cells become active killers (Virella, 2007; Wood, 2006).

Antibodies that are produced constitute a group of globular serum proteins known as immunoglobulins (Igs). There are five class of Ig; IgM, IgG, IgE, IgA and IgD. Antibody responses after primary dengue infections are largely of the IgM class and are predominantly directed against type-specific determinants. In secondary infection, IgG are the dominant class and they are directed against the antigens of the flavivirus group (Halstead, 1988).

The binding of antibodies to antigens to form antigen-antibody complexes is the basis of several antigen disposal mechanisms. There are four types of disposal mechanisms. First is neutralization. The antibody will bind to and blocks the activity of the antigen. The pathogens that are now coated with antibodies will be eliminated by phagocytosis. In a process called opsonization, the bound antibodies enhance macrophage attachment to, and thus phagocytosis of, the viruses (Wood, 2006).

Second is agglutination where the antibody will agglutinate the pathogens. These large complexes are then readily phagocytosed by macrophages. The cross-linking of soluble antigen molecules will dissolve in body fluids, thus forming immobile precipitates that are disposed of by phagocytes.

The last and most important disposal mechanism is complement fixation/system. The complement system carries out a cascade of steps leading to lysis of viruses. Some of them function with chemokines in attracting phagocytic cells to the sites of infection. Complement consists of 20 different serum proteins that are inactive. When the first component of complement, C1 is activated, it will trigger a cascade of activation steps, each component activating the next in the series. Completion of the complement cascade results in the lysis of many types of pathogenic cells. Soluble and membrane-associated NS1 have been demonstrated to activate human complement (Guzman *et al*., 2010).

There are three complement pathways. First is classical pathway. C1-C9 components are involved in this pathway. When a viral infection occur, a receptor known as Fc receptor, will bind to antibodies that are attached to virus-infected cells or invading viruses. As the antibody binds to a virus, the Fc portion of the antibody alters conformation so that it can bind to C1. Unfortunately, not all antibody classes can bind to C1. IgM is the most efficient, followed by IgG (class 1, 2 and 3). IgG class 4 (IgG4) could not bind C1. C1 has three components called C1q, C1r and C1s. There are six C1q, two C1r and two C1s subunits in a C1 molecule. To activate C1, at least two C1q are needed to bind Fc binding site. IgG molecules have only one Fc portion, which means that it needs two or more IgG molecules to activate C1 whereas IgM molecule have five Fc regions, so one molecule of IgM is enough to activate C1. When C1q undergoes a conformational change due to the bond to Fc regions, it will activate C1r and C1s (Virella, 2007; Wood, 2006).

C1s will then cleave C4 into C4a and C4b. NS1 was also reported to interact directly with C4, promotes efficient degradation of C4 to C4a and C4b (Avirutnan *et al*., 2002). C4a will activate mast cells, which will release histamine during inflammatory response. C4b will bind to C2 component and their binding will cleave C2 into C2a and C2b by C1s. C2b diffuses away but C2a remains bound to C4b and will form C4b2a complexes. The substrate of this complex is C3, which is the key component of all the complement pathways. Individual who lacks C3 suffer from recurrent, life-threatening viral infection. C3 that is been activated by the complex, will bind to the complex to cleaved into C3a and C3b. Large amount of C3a has been detected in patients with severe dengue (Nielsen, 2009). Its function is to activate mast cells, capable of disrupting vasculature,

serves to recruit monocytes, macrophages and dendritic cells, regulates vasodilation, and increases permeability of small blood vessels and smooth muscle contraction (Nielsen, 2009). C3b binds to the cell to which the antibody is bound and acts as opsonin, promoting the phagocytosis of the cell. C3b will bind to C4b2a3b complexes (Virella, 2007; Wood, 2006).

A C4b2a3b complex is a C5 convertase and cleaves C5 to C5a and C5b. C3a and C5a regulate vasodilation, increase permeability of blood vessels and can trigger degranulation and oxidative burst from neutrophils, eosinophils and basophiles (Nielsen, 2009). C5b binds to the cell surface. The cleavage of C5 is the last enzymatic step in the complement pathway. The later steps are involved in generating pores in the membrane that will result in lysis of the virus cell. These pores are known as the membrane attack complex (MAC). C5b will bind to C6, followed by C7 and C8 to form C5b678. During the binding of these molecules, some of them will undergo conformational changes that result in highly lipophilic structures in C7 and C8 inserting into the membrane. These complexes can form small pores in the pathogen membrane about 7-10 nm in diameter. This will cause ions and water to rush into the cell, causing it to swell and lyse (Virella, 2007; Wood, 2006).

The second pathway is the lectin pathway. It is similar to the classical pathway, with an exception that instead of antibody binds to an antigen on a virus; MBP binds to mannose residues on the surface of virus at the very first step. These mannose residues may be components of glycoproteins or polysaccharides. When MBP has bound to the pathogen, a

protease called mannose binding lectin (MBL)-associated serine protease (MASP) binds to the MBP. This complex will cleave C4 into C4a and C4b and also cleaves C2 into C2a and C2b, the same way C1s performs in the previous pathway. Once C4b is generated, the rest of the sequence is the same as classical pathway (Virella, 2007; Wood, 2006).

In the third pathway; alternative pathway, C1, C4 and C2 are not involved as the other two pathway mentioned above. However, C3 and C5- C9 are involved. Different components are used to generate C3 and C5 convertases.

Beyond its lytic capacity, complement protects against viral infections by priming adaptive B and T cell responses, triggering leukocyte chemotaxis through the release of anaphylatoxins (C3a and C5a), and opsonizing viruses for phagocytosis and destruction of macrophages (Avirutnan *et al*., 2011).

In cell-mediated response, class I HLA molecules are involved. As a newly synthesized molecule moves towards the cell surface, it will capture a fragment of protein containing replicating virus within the same cell and transported to the cell surface. This molecule exposes the foreign proteins that were synthesized in infected cells to T_C cells. The interaction between antigen-presenting infected cells and T_C is enhanced by the presence of T cell surface protein called CD8. This protein will bind to a portion of the class I HLA protein and helps to keep the two cells contact while activation of T_C cell is occurring. The stimulation of IL-2 from T_H cells will activate T_C cell, which will

differentiate into an active killer. It will kill the APC by releasing perforin, a protein that forms pores in the virus cell's membranes. This causes ions and water flow into the virus cell, thus causing the cells to swell and lyse. This cell also releases IL-10 during dengue infection to inhibit NK cells. Increased in IL-10 levels will reduced the levels of platelets and reduced platelet function (Libraty *et al*., 2002), which will leads to thrombocytopenia.

2.1.3 Clinical features of dengue

During the early febrile phase, clinicians cannot predict which patients will progress to severe disease. Infection with either one of the serotypes can cause a broad spectrum of manifestations from asymptomatic or mild DF to DHF or DSS.

Usually, DF is observed in primary infection, with symptoms such as body pain, high fever (frequently reaching 39°C or higher), headache, fever, maculopapular rash, retro-orbital pain (pain involves eye area), muscle (myalgia) and joint (arthralgia) pains (Kautner *et al*., 1997). Clinical laboratory findings associated with dengue fever include neutropenia followed by increasing levels of alanine transaminase (ALT) and aspartate transaminase (AST) between 500 and 1,000 units/L. Besides that, the platelet count will decrease to a measurement of lesser than 100,000/µL (Gubler, 2006). In mild cases, the same symptoms are frequently presented, with nausea, vomiting and lymphadenopathy. The acute illness may last up to one week, with prolonged convalescence for several weeks (Rivetz *et al.*, 2009).

DHF is usually associated with secondary infection. It is an acute febrile illness with minor or major bleeding, thrombocytopenia (platelet count below 50,000/ µL) and evidence of plasma leakage. Among the symptoms are bleeding manifestations (at least a positive tourniquet test), plasma leakage, circulatory collapse or shock (high pulse rate and narrowing of the pulse pressure to 20 mm Hg or less) (Rigau-Pérez *et al*., 1998). DHF commonly begin with a sudden rise in temperature, around 38°C to 40°C, and last for more than two days. In most cases, patients will develop petechiae, purpuric lesions and ecchymoses. Purpuric lesions may appear on various parts of the body but are most common at the site of venipuncture. Epistaxis, bleeding gums, gastrointestinal hemorrhage and hematuria occurs less frequently. The amount of platelet will rapidly decrease (20,000 platelets/mm^3 or less) and the haematocrit (HCT) (volume percentage of red blood cells in blood) will increase by 20 % (Kautner *et al*., 1997) , which indicate increased probability of impending shock (Nimmannitya, 1994). High concentration of hematocrit will lead to loss of intravascular volume and thus increases viscosity of blood which may result in circulatory problems.

DHF can be classified into four grades of illness based on severity. Grade I DHF is mild; whereby the only hemorrhagic manifestations are scattered petechiae or positive tourniquet test. Grade II DHF is more severe, with one or more overt hemorrhagic manifestations. The most severe forms of DSS are Grade III and IV. Grade III illness is characterized by mild shock with signs of circulatory failure. The patients maybe lethargic or restless and have cold extremities, clammy skin, a rapid but weak pulse, narrowing of

pulse pressure to 20 mm Hg or less or hypotension. Grade IV is characterized by profound shock with undetectable pulse and blood pressure (Martina *et al.*, 2009).

In DSS, patients will have the same symptoms as DHF, including signs of circulatory failure, hypotension and frank shock. Rigau-Pérez and co-workers (1998) noted that the signs for impending shocks are intense, sustained abdominal pain, persistent vomiting, restlessness or lethargy and a sudden change from fever to hypothermia with sweating and prostration (Rigau-Pérez *et al*., 1998). According to Halstead *et al.* (2005), the phenomenon of Antibody Dependent Enhancement (ADE) theory postulates that the infection with one dengue serotype during primary infection confers future protective immunity against that particular serotype but not with other serotypes during a secondary infection (Halstead, Heinz, Barrett, & Roehrig, 2005).

2.1.4 Diagnosis of dengue virus infection

There are two parts of laboratory investigations to identify patients with dengue infection and these are the (i) disease monitoring laboratory tests and (ii) diagnostic test.

In the disease monitoring laboratory tests, the patients are tested based on their full blood count (FBC) and liver function test. The FBC covers the white cell count (WCC), HCT and thrombocytopenia. If the WCC of the patients decrease rapidly compared to healthy individuals, it shows a trend of leucopenia, where there is a possibility the patients

might have dengue infection. One of the symptoms of plasma leakage in dengue infection is the rise of HCT. This rise helps to differentiate between DF and DHF but it can be masked in patients with concurrent significant bleeding and in those who receives early fluid replacement. To recognize the rising of HCT level, the patient's HCT baseline should be set in the early febrile phase of disease (Ministry of Health (MOH) Malaysia, 2010). Thrombocytopenia is one of the symptoms commonly seen in dengue infection. Platelet count is usually at the normal level in the early febrile phase. However, as the disease progress, it will decrease rapidly and may continue to remain low for the first few days of recovery. In liver function tests, elevated liver enzymes is common and is characterized by greater elevation of the AST as compared to the ALT. DHF patients have higher frequency and degree of elevation of the liver enzymes compared to DF patients (MOH Malaysia, 2010).

There are three types of diagnostics test that could be performed for the confirmation of dengue infections: - dengue serology test, virus isolation and polymerase chain reaction (PCR).

Various serologic tests are available to detect antibodies against dengue, including the hemagglutination inhibition (HI) assay, the complement fixation (CF) test, the enzyme-linked immunosorbent assay (ELISA) and viral neutralization test.

HI test was used to differentiate between a primary and secondary infection of dengue. In primary infection, antibody titer is generally less than 1:20 in a sample collected within the first four days after the onset of symptoms. Patients with the HI titre of equal or higher than 1:2560 are considered as having secondary infection (Gubler, 1998). However, there are limitations when using HI. It is difficult to interpret if the interval between the acute and the convalescent phase samples is less than 7 days, or convalescent phase samples are not available (Gubler, 1998). Moreover, other difficulties using HI tests includes: difficulty to standardize, lack of specificity, it is not applicable for primary infection diagnosis, the need for paired samples, time consuming and could not identify the infecting virus serotype (Paula & Fonseca, 2004).

The complement fixation (CF) test is based on the principle that the complement will be consumed during the antigen antibody reaction. In CF, the antibodies that were detected appear slower than HI antibodies and it only lasts for short periods (Paula & Fonseca, 2004).

Detection of antibodies, especially the IgM antibody capture ELISA (MAC-ELISA) is the most widely used test in dengue diagnostic. This assay is based on detection dengue-specific IgM antibodies in the test serum by using anti-human IgM antibody previously bound on a solid phase. Whenever an infection occurs, IgM antibody will be the first immunoglobulin isotype to appear. IgM antibodies rise by the third day of the onset of symptoms and may be present for three months (Rivetz *et al.*, 2009). However, by the end of the first week of disease onset, anti-dengue IgG will appear in a low titre and increases slowly. Therefore, the detection by MAC-ELISA in a single specimen is an indication of an

active or recent dengue infection (Seah, Chow, Chan, & Doraisingham, 1995). However, this assay is able to detect dengue IgM in up to about 80% of acute phase sera of known dengue patients. Another group reported that detection of IgM could not be used to determine the serotype of dengue mainly due to the cross-reactivity of an antibody observed during primary infection (Guzmán & Kouri, 2004).

During a secondary infection however, IgG antibody titre will rise rapidly and the antibody will react broadly with other flavivirus. It remains in the immune system for many years. An ELISA for dengue-specific IgG detection can be used to confirm a dengue infection in paired sera. According to Innis *et al.* (1989), the ratio of dengue virus IgM antibodies to the dengue virus IgG antibodies can determine the classification of either primary or secondary dengue infections (Innis *et al.*, 1989). Patients with IgM/IgG ratios higher than that of 1.78 are considered as having primary dengue virus infection, while those with lower than that value are considered as secondary dengue virus infection (Innis *et al.*, 1989). IgG antibody are usually measured by ELISA and the HI test, as discussed earlier.

The plaque reduction neutralization test (PRNT) allows for virus-antibody interaction to occur in a microtiter plate. The antibody effects on viral infectivity are measured by plating the mixture on virus-susceptible cells. Prior to this, the samples that are being tested are usually subjected to serial dilutions with a standardize amount of virus. This is done so that individual plaques can be discerned and counted when the virus are

added to susceptible cells and overlaid with semi-solid media. This media restrict the spread of progeny virus. Each virus that initiates a productive infection produces a localized area of infection (a plaque). To determine the percent reduction in total virus infectivity, the plaques are counted and compared back to the starting concentration of the virus. In this way, PRNT end-point titers can be calculated for each serum specimen at any selected percent reduction of virus activity (WHO, 2007). To date, this test is widely used for immunity studies. It is also the most serologically virus-specific test among flaviviruses, and serotype-specific test among dengue viruses, correlating well to serum levels of protection from virus infection. However, due to its time-consuming nature, labor intensiveness and low throughput, this test is not routinely used for dengue diagnostics (Tan & Ooi, 2012).

According to the clinical practice guidelines by the Ministry of Health Malaysia, virus isolation is the most definitive test for dengue infection. Only samples at the early phase, *i.e.* before day 5 of illness, are useful for this test to avoid from the formation of neutralizing antibodies. Dengue virus can be isolated from serum, plasma and leukocytes during the febrile stage of illness and also from post mortem specimens. In dengue isolation, the *Aedes albopictus* mosquito C6/36 cell line is used. After an incubation period permitting virus replication, viral identification is performed using the monoclonal antibody immunofluorescene (IF) test and PCR assays. However, the limitation of this test is that it is very expensive and may take up to two weeks to be completed.

Another alternative method for diagnosis is via detection of viral RNA. This is performed by using reverse transcriptase-PCR (RT-PCR) and real-time RT-PCR. The most commonly used RT-PCR are based on single RT-PCR assay, a nested RT-PCR assay and one-step multiplex RT-PCR assay. The nested PCR reaction involves an initial reverse transcription and amplification step using dengue primers that target a conserved region of the virus genome followed by a second amplification step that is serotype specific. Following that, the products of these reactions are separated by electrophoresis on an agarose gel which allows the dengue serotypes to be differentiated on the basis size (Guzman *et al*., 2010).

Compared to the nested RT-PCR approach, the real-time RT-PCR is considered to be more sensitive and could detect as many as four more times acute-phase samples (Poersch *et al.*, 2005). This real-time RT-PCR is a one-step assay that allows virus titre to be quantified in approximately 1.5 hours (hrs) (Guzman *et al*., 2010). The detection of the amplified target by fluorescent probes replaces the need for post-amplification electrophoresis. TaqMan and fluorogenic probes were designed to identify the different dengue serotypes, resulting in high sensitivity and specificity (Chutinimitkul, Payungporn, Theamboonlers, & Poovorawan, 2005). Further development in PCR detection chemistry resulted in the use of fluorogenic dyes such as SYBR® Green. The SYBR® Green method can be directly applied to any gene without the need to design and synthesize fluorescently labeled target-specific probes. It has been used by many researchers to detect DENV RNA in human samples for all serotypes (Chutinimitkul *et al*., 2005; Johnson, Russell, & Lanciotti, 2005; Leparc-Goffart *et al*., 2009; Yong, Thayan, Chong, Tan, & Sekaran, 2007).

SYBR® Green is used as fluorescent indicator as it could easily detect the amplified products due to its ability to intercalate in the minor groove of double-stranded deoxyribonucleic acid (DNA). The percentages of guanine and cytosine (G+C) content in each dengue genotype are different, which makes the melting temperature (Tm) values differ from each other. Thus using this technique, all four types of dengue virus can be identified based on its distinctive Tm values. Papin and collegue in 2004 reported that the use of SYBR® Green is more reliable, flexible, simpler and cost lower compare to other PCR method (Papin, Vahrson, & Dittmer, 2004).

2.1.5 Dengue in Malaysia

Dengue is endemic in Malaysia where the first dengue case was reported in 1902 in Penang (Skae, 1902) and the first major outbreak of DHF was reported in 1973 with DENV-3 implicated as the dominant serotype (George, 1992). In 1982, a large dengue epidemic was reported with 3,005 notified cases, of which 28.4 % were DHF. According to the Ministry of Health Malaysia, 20,837 dengue cases were reported in 2011 (Ministry Of Health Malaysia, 2012), with 34 deaths.

The number of cases of DF and DHF reported in Malaysia shows an increasing trend from 1995 to 2007 (Ministry Of Health Malaysia, 2012). However, there was a decrease of both cases from 1998 to 2000, before the cases started to increase back the following year. It was found that the incidence rate was higher in the age group of 15 years and above, which involved the working and school-going age groups. The same group has been observed to have an increase of dengue deaths since 2002. Most of the dengue cases

reported were from urban areas (70 – 80 %) where there is a high density of its population and rapid development activities which are factors that favour dengue transmission.

2.1.6 Management of dengue

Presently, there are no drugs or licensed vaccines available for the management of dengue (Whitehorn & Farrar, 2010). Dengue vaccine development is complicated due to the need to incorporate all four virus serotypes into a single formulation (Ooi & Gubler, 2008). There is also no specific treatment for DF or DHF, therefore patients are usually treated according to the symptoms they presented.

According to Rigau-Perez *et al.* (1998), patients with DF are usually required to rest and are given oral fluids and electrolyte therapy to compensate for fluid loses as a consequence of diarrhea or vomiting. Paracetamol or any analgesics and antipyretics are needed to control the high fever. However, the use of aspirin and ibuprofen must be avoided as it could induce gastric irritation and severe bleeding. Aspirin must also be avoided to prevent Reye's syndrome which is associated with dengue infection (Cook & Zumla, 1999).

The major problem in DHF/DSS is fluid loss rather than blood loss. So, the patient's management must be directed toward maintenance of their blood volume and blood pressure. Patients that show shock symptoms should be given fluid replacement (crystalloids) to avoid haemodynamic instability, narrowness of blood pressure and

hypotension. Intravenous (IV) fluids were given in order to overcome plasma leakage. In mild to moderate severe cases (grades I and II), fluid therapy can be given for a period of 12-24 hr at an out-patient clinic where there are facilities to monitor vital signs and HCT. Patients who continue to have high HCT or present with any signs should be admitted to the hospital. When dengue shock becomes prolonged or recurrent, IV fluids should be given carefully according to age and dosage to prevent fluid overload as this could result in pulmonary oedema (Guzman *et al.,* 2010). Overload of fluid will also produce massive effusions, respiratory compromise and congestive heart failure. Insufficient volume replacement will allow the worsening of shock, acidosis, and disseminated intravascular coagulation. Patients that have a great loss of plasma must be given isotonic solutions and plasma expanders.

The patient's vital signs should be measured every 30-60 min and HCT level every 2-4 hr. As the patient's condition start to stabilize, the measurement may be taken at lesser frequency. Patients should at least be monitored for a day after the fever started to drop. Once the patient begins to recover, IV fluid is rapidly reabsorbed, causing a drop in HCT. The patients should meet the following criteria before being discharge: absence of fever for 24 hr without any medication, return of appetite, stable HCT and has a platelet count that is greater than 50 000 /µL.

The management of the environment is also an important factor to consider in order to control and prevent dengue infection. Mosquitoes breed primarily in stagnant water that

accumulate in water storage container or vessel, flower vases, old jars, tin cans, used tyres in and around human dwellings. Citizens should participate in the cleaning-up campaign, where the water containers should be cleaned and emptied to eliminate breeding sites. The government should impose stricter enforcement to deter citizens from allowing *Aedes* to breed in their housing area or compounds.

2.2 The use of traditional medicine in treatment of dengue

2.2.1 General

The use of traditional herbal remedies is mostly used in developing countries, where they are still relying on plant-based medicine for their health care. This is because they find that these natural products are effective, have fewer side effects and has relatively low cost.

In West Africa, the pounded bark of *Trichilia emetica* (Natal Mahogany) is used to treat parasitic skin infections and inflammation. Tribal medicinal practitioners in Bangladesh used *Clerodendrum viscosum* (hill glory bower) leaf to treat frequent fever, malaria fever and dengue fever (Rahmatullah *et al*., 2011). The essential oil from leaves of *Lantana camara* (wild sage) has been reported by Dua, Pandey and Dash (2010) to have adulticidal activity against the dengue mosquito, *Aedes aegypti*. The essential oil from *Lippia alba* (Bushy Lippia) was reported by Ocazionez, Meneses, Torres, and Stashenko (2010) to have anti-viral activity against dengue virus replication. The leaves and flowers extracts of *Nyctanthes arbor-tristis* (Night-flowering Jasmine) were shown to have larvicidal activity against the *Aedes aegypti* (Mathew *et al*., 2009).

In Pakistan, a popular natural remedy to treat dengue is to use juice of apple with some drops of lemon to increase the level of platelets in patients' blood (Anees, 2011).

The aboriginal people from Kampung Bawong, Perak, used local plants such as the leaves of *Sambucus javanica* (Chinese elder), *Sansevieria trifasciatai* (snake plant) and the whole plant of *Stachytarpheta jamaicensis* (blue porterweed) to reduce pain and inflammation (Samuel *et al*., 2010). It was reported that this tribe used the whole plant of *Gnetum leptostachyum* and *Ardisia colorata*, the leaves of *Abutilon indicum* (Indian Mallow) and *Bulbophyllum mutabille* and the flowers of *Aglaia odorata* (Chulan) to relieve flu and fever. Young leaves of *Cnestis platantha* (garing-garing) was used to treat high fever.

2.2.2 *Carica papaya*

Carica papaya or its common name, papaya, belongs to the Caricaceae family. The plant originates from Central America and could be found in all tropical countries as well as many subtropical regions of the world. While the leaves of this plant are commonly used as vegetable, its fruit extracts was used as therapeutic remedy due to several of its medicinal properties (Mahmood, Sidik, & Salmah, 2005). In India, unripe cooked fruits are frequently taken to encourage lactation (Sayed, Deo & Mukundan, 2007). The hospitals in Africa used the papaya pulp for treating wounds and burns (Starley, Mohammed, Schneider & Bickler, 1999).

In India, the seeds has been used as a potential post-testicular anti fertility drug (Lohiya, Pathak, Mishra, & Manivannan, 2000). Due to the anti-helmintic activity shown by the latex and seeds, these two parts has been used in the care of gastrointestinal nematodes infections.

CPL extract has been traditionally used to treat stomachache, symptoms of asthma, fever and gastric problems. In addition, CPL extract has been shown to accelerate wound healing process in rats (Mahmood *et al*., 2005). *Carica papaya* has also been reported by Lampiao (2011) to have antifertility effects. The extract of CPL has shown to cause a drop in sperm motility and pH of semen in rabbit and human reproductive system (Nassar, 1979). Ahmad *et al.* (2011) and Subenthiran *et al.* (2013) reported that the level of platelet count increased in dengue patients after consuming CPL for five and three consecutive days, respectively. The leaves were also effective against the oxidative damage caused by lead acetate in the bone marrow and had a stimulatory effect on haemopoiesis (Tham, Chakravarthi, Haleagrahara & Alwis, 2013).

2.2.3 Composition of *Carica papaya* fruit and leaves

The papaya fruit contains immuno-stimulating and antioxidant agent (Aruoma *et al*., 2006). The unripe fruit contains the enzyme papain, a cysteine protease with an action similar to that of pepsin in gastric juice (Thomas *et al.*, 2009). This enzyme is used as meat tenderiser, clarifier in beers and in treatment for osteoporosis, arthritis, vascular disease and cancer (Adaikan & Adebiyi, 2004). Ncube, Greiner, Malaba and Gebre-Medhin (2001) reported

that β-carotene supplementation with yellow-to-orange fruits (papaya) improves the vitamin A and iron status of lactating women.

The leaves extract on the other hand, has been reported to contain many active compounds such as ascorbic acid, α-tocopherol, chymopapain, cystatin, papain, flavonoids, cyanogenic glucosides and glucosinolates that can increase the total antioxidant power in blood and reduce lipid peroxidation level (Otsuki *et al.*, 2010). CPL has also been traditionally used for a long time to treat cancer and infectious disease. In Australia, CPL juice has been consumed as a supplement by the aboriginal community for its anticancer activity (Otsuki *et al*., 2010).

2.3 Proteomics

2.3.1 Overview of proteomics

Proteomics is the study of proteins, their structures, localization, post-translational modifications, functions and interactions with other proteins (Lee, Han, Altwerger, & Kohn, 2011). Proteomics technologies can be used in the identification of new targets for therapeutics, development of novel biomarkers for diagnosis and early detection of disease; and is also used in clinical trials for drug discovery and development (Burbaum & Tobal, 2002). There are two types of strategies that are commonly utilized in proteomics, which are the gel-based and the gel-free methods.

2.3.2 Importance of proteomics

Proteomics are generally used for proteome profiling, for comparative expression analysis of two or more protein samples, for the localization and identification of post-translational modifications, and for the study of protein–protein interactions (Chandramouli & Qian, 2009). It is a research field that gathers environmental and genetic factors, and the 'proteome' represents the functional status of a biological compartment (Monteoliva & Albar, 2004). Genomics, on the other hand is the study of genes in an organism. Among the study involves in genomics are the study of the structure and roles of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) which led to the development of molecular genetics and the variations in nucleotide sequences at different physical locations (loci) in the genome of an individual (Edmeades, McMaster, White & Campos, 2004) .

2.3.3 Techniques for proteomics

The gel-based method involves the separation of complex mixtures of protein using 2-dimensional gel electrophoresis (2D-GE). Proteins are first separated according to their isoelectric point (p*I*) (net charge) at different pH values by isoelectric focusing (IEF). Subsequently, the protein will undergo another separation based on their molecular weight (MW) in the second dimension. The combination of both types of separation makes it possible to obtain an almost uniform distribution of protein spots across a 2D gel. 2D-GE analysis has facilitated the rapid characterization of thousands of proteins in a single polyacrylamide gel. It may provide several types of information including quantity, molecular weight, p*I* and possible post-translational modifications (Chandramouli & Qian, 2009). The gel-based workflow is mostly used for qualitative experiments. It is a simple

method that enables visualization for mapping differences in protein expression. The overview for a 2D-GE workflow is represented in Figure 2.

There are however, some limitations to 2D-GE in which the staining method may not be able to visualize all proteins in a complex sample. Only 30-50% of the entire proteome, depending on the type of tissue can be visualised (Baggerman, Vierstraete, Loof & Schoofs, 2005). The strips used are important to enable visualization of the protein of interests. The strips chosen must be based on the types of cells and also the relative focusing power. This relative focusing power expresses the enhanced resolution expected in the first dimension using strips with different lengths or pH ranges. As an example, when two strips have the same pH range but different in the strip length, the ratio of the strip lengths gives the relative focusing power of the longer strip. On the other hand, two strips with the same length but different pH range, the ratio of the pH ranges gives the relative focusing power of the strip with smaller range.

2D-GE also has limited ability to study certain classes of proteins such as ribosomal proteins, some RNA binding proteins and translation factors (Abdallah, Dumas-Gaudot, Renaut & Sergeant, 2012). Besides that, it cannot detect low abundant and hydrophobic proteins, has low sensitivity in identifying proteins with either pH lower than 3 (pH<3) or pH higher than 10 (pH>10) and either small or large molecular masses (lesser than 10kDa and bigger than 150kDa) (Chandramouli & Qian, 2009).

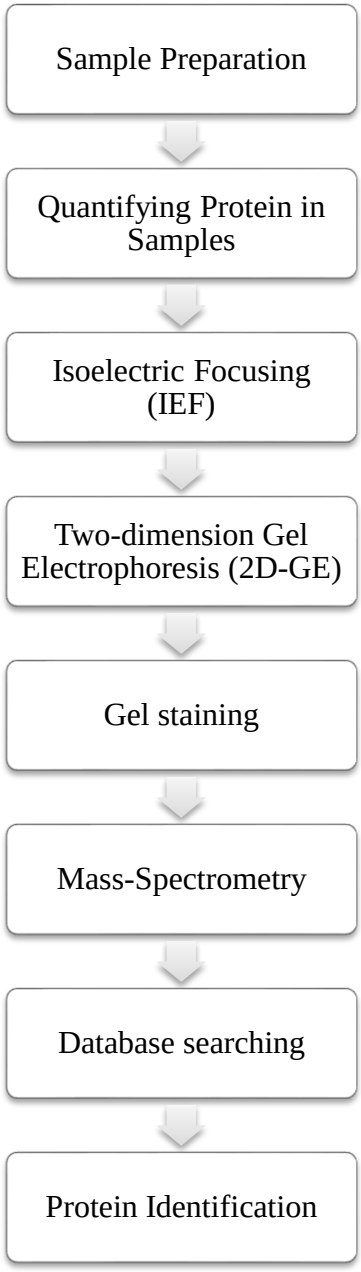

Figure 2: Workflow for 2D-GE

Walton and Jayaraman (2009) reported a technology where it enriches and separates low abundance proteins in complex samples. The technology known as ProteoMiner technology depletes higher abundance proteins while enriching lower abundance proteins in samples where the proteins span a large dynamic range. Alternatively, the Albumin and IgG Removal Kit (GE Healthcare) which improves resolution of low-abundance proteins and increases the number of spots in the treated sample is also available.

Nevertheless, 2D-GE is still favoured by many researchers and it is currently the most rapid method for direct targeting of protein expression differences. It is also the most widespread strategy for comparing distinct state of two proteomes since the development of immobilized pH gradient strip (Monteoliva & Albar, 2004).

The gel-free proteomics techniques that are usually utilised is the multi-dimensional capillary liquid chromatography coupled to tandem mass spectrometry (MDLC-MS). It separates and identifies the peptides obtained from the enzymatic digest of an entire protein extract (Baggerman *et al*. 2005). Proteins are cleaved into peptides using proteolytic enzymes and these peptides are separated and subjected to tandem mass spectrometric analysis for identification. It has become a method of choice for the analysis of membrane and low-abundant protein and thus could be used to complement the 2D-GE analysis. Liquid chromatography (LC)-based methods are more effective for the analysis of small proteins and peptides, membrane and highly hydrophobic protein (Thongboonkerd, 2007). A peptide based proteomics analysis can be performed at a much faster rate and cost cheaper compared to a complete gel-based analysis. However, this technique has a

limitation whereby it is difficult to perform differential display analysis (Monteoliva & Albar, 2004).

2.3.4 General gel-based proteomic flow

2.3.4.1 Sample preparation for 2D-GE

This step is done to ensure only proteins are isolated when processing a sample of interest, and at the same time to eliminate all other biological compounds that might interfere with the first separation. The charge of proteins must not be altered in any way during this process. Substances such as high concentrations of salt ions, nucleic acids, lipids, polysaccharides, charged metabolites and impurities in the samples should be completely removed or minimized so that all the intracellular proteins can be fully analysed (Penque, 2009). The process involved includes disrupting, inactivating or removing interfering substances and followed by solubilisation to ensure that only the majority of proteins in the sample will be obtained by the end of the experiment. Different set of proteins might be obtained when using different methods of sample preparation.

According to Burtis and Ashwood (2001), the concentration of human serum; with the addition of small molecules such as salts, lipids, amino acids, and sugars; is in the range of 60–80 mg of protein/ml after the removal of clotting factors. The major protein constituents of serum include albumin, immunoglobulins, transferrin, haptoglobin, and lipoproteins. 50–70% of the total protein in a human serum constitutes albumin and 10–25% is IgG (Adkins *et al*., 2002). The presence of these proteins obscures other proteins in

the gel and limits the amounts of proteins in the serum that can be resolved by 2D-GE. These proteins also have wide range of p*I* and molecular weight that further reduce resolution of separation and mask some low-abundance proteins. Thus, the removal of high abundant proteins from serum or plasma samples is routinely the first step taken for clinical proteomics analyses aiming at biomarker discovery.

In this study, the ProteoMiner™ kit was utilised as this technique uses a bead-based combinatorial peptide library of ligands to bind proteins in a non-targeted manner. High abundance protein will rapidly saturate the binding capacity of their designated bead ligands while low abundance proteins do not, resulting a majority of the high abundance proteins being unable to bind and washed out, leaving the remaining low abundance protein until the beads were eluted off. Figure 3 shows the diagrammatic representation of this process.

2.3.4.2 Quantification of protein samples

Protein samples need to be quantified so that an appropriate amount of protein is loaded. Accurate quantitation facilitates comparison between similar samples by allowing identical amounts of protein to be loaded. When using a strip of 11 cm length, 250 µg of protein is usually loaded (Ramagli & Rodriguez, 1985).

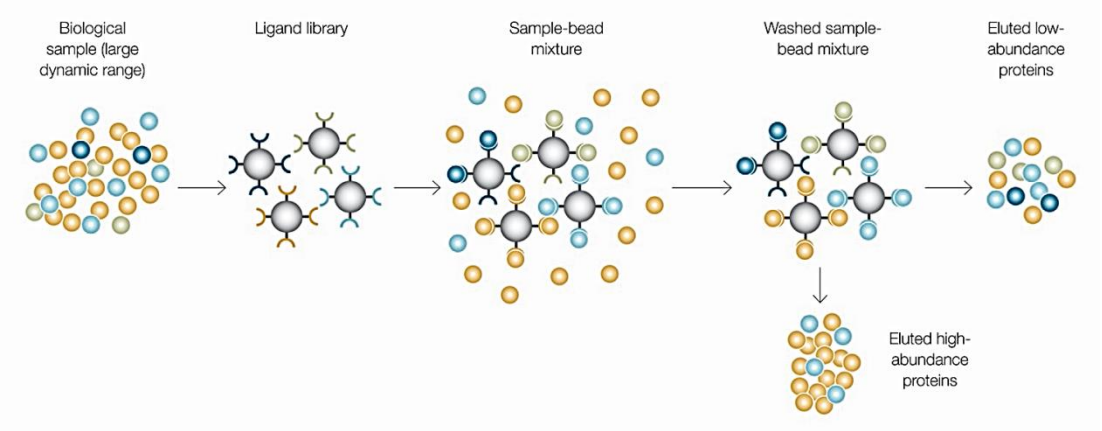

Figure 3: ProteoMiner™ technology.

In ProteoMiner™ technology, each bead features a different hexapeptide ligand with affinity for specific proteins in a sample. Samples are applied to the beads, allowing proteins to bind to their specific ligands. Excess proteins are washed away, and those proteins that bound to the beads are eventually eluted, allowing further downstream analysis. (Adapted from http://www.genengnews.com/gen-articles/dementia-studies-making-strides/3877/?page=2)

2.3.4.3 First-dimension isoelectric focusing (IEF)

IEF is a technique where by proteins are separated according to their p*I i.e* the point where their charge is the same as the surrounding pH (Garfin, 2003). The sum of all positive and negative charges of any particular protein is the net charge of that protein. The net charge is determined by the ionisable acidic and basic side chains of the constituent amino acids and prosthetic groups of the protein. A protein is classified to be acidic when the number of acidic groups in that protein exceeds the number of basic groups, which will lead the p*I* of that protein to be at low pH value. Proteins are classified to be basic when the basic group exceed the acidic groups in a protein, thus the p*I* will be high. Proteins approach their respective p*I* values at different rates but remain relatively fixed at those pH values for extended periods.

Proteins are positively charged at pH values below their p*I* and negatively charged at pH values above their p*I*. Proteins with pH value below their p*I* will migrate toward the cathode during electrophoresis. However, the proteins will move towards the anode if the pH values above its p*I*. A protein at its isoelectric point will not move in an electric field (Görg, 2004).

2.3.4.4 Two-dimension gel electrophoresis (2D-GE)

The protein mixtures are separated according to their molecular mass. According to Thayan *et al.* (2009), this technique can display, quantify and identify thousands of proteins in

single gel. It can also be used to identify and differentiate protein profile of between healthy individuals and diseased individuals.

This technique is performed using polyacrylamide gels containing sodium dodecyl sulfate (SDS). When proteins are treated with both SDS and a reducing agent, the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular weight of protein. This is because reducing agents are able to break any disulphide bonds present in the proteins (Görg, 2004).

2.3.4.5 Gel staining

Once the 2D-GE is completed, the gel will be stained in order to visualize the protein spots obtained. A protein-specific, dye-binding or color-producing chemical reaction must be performed on the proteins within the gel. Among the staining techniques that are usually used are the Coomasie dye staining, silver staining and zinc staining.

Coomasie dye staining is a simple and convenient method because it involves a single, ready-to-use reagent and does not permanently or chemically modify the target proteins. Silver staining is 100 times more sensitive compared to Coomasie dye staining. It is a sensitive non-radioactive technique. The high sensitivity of this visualization technique allows detection of most proteins down to the nanogram range (Steinberg, 2009). The zinc staining technique stains all areas of the polyacrylamide gel in which there are no proteins. Zinc staining is also sensitive as it can detect less than 1 ng of protein and there are no

fixation steps. Furthermore, this stain is easily removed, making this method compatible with mass spectrometry or Western blotting.

2.3.4.6 Mass spectrometry (MS)

Proteins spots of interest that are separated during the 2D-GE will be excised, enzymatically digested, and the resulting mixture of peptides are subjected to MS analyses for the identification of proteins. The main components of MS are an ion source, mass analyser(s), and a detector. Ion source is a device that brings the analytes into gas phase and ionizes them. Mass analyser is the central component that measures the mass-to-charge ratio (m/z) of the ionized analytes while the detector functions to register the number of ions at each m/z value. The MS data are bio-informatically compared with an appropriate database to identify the origin of the protein (Liumbruno, 2008).

Matrix assisted laser desorption ionization (MALDI) induces the sample to sublimate out of a dry crystalline matrix. Gaseous ions formed by MALDI are accelerated into the mass analyser by an electric potential and the motions of the ions through the mass analyser determined the m/z ratios. The detector converts the stream of ions into a voltage that is interpreted by a computer and converted to a mass spectrum.

There are few types of mass analysers used in proteomics studies, which are the quadrupole (Q), time-of-flight (TOF), ion trap (IT), Orbitrap, linear ion trap (LIT), and Fourier transform ion cyclotron resonance (FTICR). The Q analyser transmits ions with a

narrow m/z range and uses the stability of the trajectory to separate them according to their m/z ratio on four parallel cylindrical metal rods. The TOF analyser separates ions based on the differences in transmit time, which is the time of flight, from the ion source to the detector in tubes under vacuum. The IT analyser captures or traps the ions and then subjected them to MS or MS/MS analysis. Orbitrap provides high resolution, high-mass accuracy and good dynamic range. Ions of a particular m/z in LIT are selected in a first section, fragmented in a collision cell, separated and captured in the third section, where they are excited through resonant electric fields and scanned out creating the tandem mass spectrum. FTICR operates under high vacuum in a high magnetic field.

2.3.4.7 Database searching

Once the sequence of peptide has been identified through MS, the protein is identified by comparing the peptide mass fingerprint with peptide masses obtained from theoretical digestion in protein sequence databases. Using a database search, candidate proteins are ranked from a list of most closely matched candidates using various scoring algorithms. Among the databases that can be used are Swiss Prot, the Expasy, National Center for Biotechnology Information (NCBI) and Mascot. As an example, in Mascot, the experimental mass values are compared with calculated peptide mass or fragment ion mass values. This calculated mass values are obtained by applying cleavage rules to the entries in a comprehensive primary sequence database. The closest match or matches can be identified by using an appropriate scoring algorithm. If the "unknown" protein is present in the sequence database, then the aim is to identify or match the precise entry. If the sequence

database does not contain the unknown protein, then the aim is to match entries which exhibit the closest homology, often equivalent proteins from related species.

2.3.5 Proteomics in virology research

There are many researches that have used proteomics approach in order to study virus-related disease. Wiederin, Rozek, Duan & Ciborowski (2009) has used proteomics to analyse sera of HIV-infected individuals with or without cognitive impairment. They had identified two differentially expressed proteins, prealbumin and gelsolin as potential early biomarkers in human immunodeficiency virus-induced neurodegenerative disorders. Isobaric tags for relative and absolute quantitation (iTRAQ)-coupled 2D LC–MS/MS proteomics approach was used by Feng, Wang & Chen (2010) to analyze the hepatitis B virus-replicating hepatoma HepG2 cell line. They had successfully identified apolipoprotein A-I and alpha-2-HS-glycoprotein as prognostic biomarker candidates for hepatitis B virus-associated hepatocellular carcinoma (HCC).

A study in China has been done to observe the structural and functional of lepidopteran baculoviruses progeny phenotypes, the occlusion derived virus (ODV) and the budded virus (BV). They used multiple types of proteomics approach such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE), hydrophilic interaction chromatography (HILIC) fractionation of peptides for shotgun proteomic analysis and iTRAQ labeling and Strong Cation exchange (SCX) fractionation of peptides for quantitative proteomic analysis (Hou *et al.,* 2012).

Recently, a group of researchers from Iran has used 2D-GE to investigate the neuroblastoma proteome profile in rabies viruses. They had found out that the virulent strain of rabies virus induces significant expression changes in the vimentin and actin cytoskeleton networks of neurons, thus it might have potential roles in rabies virus pathogenesis (Zandi *et al.,* 2013).

CHAPTER 3

MATERIALS AND METHOD

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3.1 Clinical materials and assays

3.1.1 Patients samples and control

Dengue patients (n=100) were recruited from Hospital Tunku Ampuan Rahimah, Klang (HTAR). The patients were recruited between April 2011 and December 2011, with written consent. The inclusion criteria of the patients to enroll in this study are (i) male or female aged between 18 to 60 years (either Malaysians or Foreigners), (ii) patients with dengue DF and (iii) the platelet counts are lesser than 100 000/µl upon recruitment. Exclusion criteria on the other hand includes: (i) pregnant or lactating women, (ii) patients with DHF grade III and IV or patients with dengue syndrome shock symptoms, (iii) patients who received blood or blood products transfusion during the current hospital stay and (iv) patients with underlying co-morbids. For all samples that were collected, the dengue virus infection was confirmed by using Dengue Duo Rapid Test that detects dengue NS1 Ag and dengue IgG/IgM.

3.1.2 Treatment

The juice used for this treatment was prepared by the Phytochemistry Unit from the Institute of Medical Research. CPL of the *sekaki* variant was chosen for the study based on fingerprinting studies and safety analysis comprising determination of heavy metals and microbial content that are within the allowable limit. For the purpose of the study, a private plantation in Semenyih, Selangor was identified to provide the leaves for the entire duration of the study to ensure similar source of authenticated raw material used. The trees in this

plantation were certified by the Ministry of Agriculture as free of herbicides, pesticides, and insecticides. Two mature CPL (approximately 50 grams) was used for the preparation of one dose of patient's treatment. The thick veins of the CPL were removed leaving only the green leaves that were then blended using a juice extractor without any addition of water. The procedures for preparing the CPL juice were performed under clean conditions.

Fifty out of 100 patients that were recruited for this study were randomly selected and were given CPL juice for three consecutive days. This group was named as Intervention group (I). Another 50 selected patients were not given any juice and this group was named as the Control group (C). After all screening procedures have been performed on the morning of recruitment, the patient's blood from both group were taken (pre-treatment/D1). The group I patients were given the 30 ml of CPL juice 15 minutes (mins) after lunch with some sweets. The blood was then taken on the next day during afternoon (post-treatment/D2) for both the I and C groups. The juice was given on for 3 days consecutively for group I, at approximately the same time starting from day 1 (D1). On day 3 (post-treatment/D3), the blood was taken from both groups of the I and C patients in the afternoon.

3.1.3 Serum sample

After the treatment procedure was performed on day 1, day 2 and day 3, blood samples were drawn from each respective patient. About 3 ml of blood were taken and collected in a plain tube. The blood was left to clot at room temperature for about one hr before it was

centrifuged at 3000 rpm for 5 min to separate the serum. The serum obtained was aliquoted in a 2 ml sterile microcentrifuge tubes and kept in -80 °C till use.

The blood taken prior treatment on day 1 (pre-treatment/D1) was used to determine the dengue serotype and in the proteomics experiments as pre-treatment group. Day 2 and day 3 bloods were used in proteomics experiments as post-treatment group (24 hrs and 48 hrs after consuming juice). Day 3 blood was also used for antibody IgM detection.

3.1.4 Ethical approval

Written informed consent was obtained from the patients prior to clinical trial participation. The protocol, patients information sheet and patients consent forms was approved by the Medical Review and Ethical Committee of the Ministry of Health prior to initiation of the study (Registration number: NMRR-09-883-4768).

3.2 Methodology

3.2.1 Determination of dengue status

(a) SD BIOLINE™ Dengue NS1 Ag-Ab Combo Kit

Dengue IgM and IgG antibodies were measured with the commercially available SD BIOLINE™ Dengue Duo NS1 Ag and IgG/IgM Rapid Test. The assay was

performed according to the manufacturer's instruction and results are classified as positive, negative and equivocal.

(b) Dengue virus PCR and serology

i) RNA Extraction

Dengue viral RNA was extracted from 140 µl of serum using the QIAmp® viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's procedure and stored at -80°C before used.

ii) Real-time RT-PCR

The reaction mixture consisting of 5.0 µl of RNA, 12.5 µl of 2XQuantiTect SYBR® Green PCR Master Mix (QIAGEN, Hilden, Germany), 10 µM Dengue Forward Primers, 10 µM Dengue Reverse Primers, and 0.25 µl Quantitect RT® was prepared. Nuclease-free water was added to the mixture to bring it to a final volume of 20 µl. The real-time PCR amplification was carried out in a Rotor-Gene-Q (Qiagen, GmbH, Germany).

Table 1: Recipe for preparation of the SYBR® Green Real-Time RT-PCR master mix

Reagent mix		**1x**
Master Mix	Nuclease Free Water	6.25 µl
	2x Quantitect SYBR® Green	12.5 µl
	10 µM Forward Primer UTR_DENF	0.5 µl
	10 µM Reverse Primer UTR_DENR	0.5 µl
	QuantiTect RT (Enzyme)	0.25 µl
Template		5.0 µl
Final Volume		25.0 µl

Table 2: Sequence of primers for real-time RT-PCR SYBR® Green

Dengue Primer	**Sequences**
UTR-DEN-F (Forward)	Sequence (5'-3') TTA GAG GAG ACC CCT CCC
UTR-DEN-R (Reverse)	Sequence (5'-3') TCT CCT CTA ACC TCT AGT CC

The thermal cycling conditions used are as follows:-

Reverse transcription was performed at 50°C for 30 min followed by initial denaturation at 95°C for 15 min. This was followed by 40 cycles of PCR with the following steps: denaturation at 95°C for 20 seconds (sec), annealing at 55°C for 30 sec and extension at 72°C for 40 sec. Upon completion of the last PCR cycle, a final extension at 72°C for 7 min was performed. This was followed by melt curve analysis which was set to begin at 75°C with an interval of 5 sec for every 1.0°C increase, until 95°C.

The specificity of the PCR product was analyzed by looking at its Tm. The Tm for each sample was used to identify the dengue serotype and the samples sharing the same Tm were interpreted as belonging to the same serotype (Yong *et al*., 2007).

(c) Enzyme-Linked Immunosorbent Assay (ELISA)

All samples were further confirmed for DENV infection serologically using a capture IgM-ELISA kit by Panbio Dengue IgM Capture ELISA (Inverness Medical Innovations, Australia). All the procedures done were based on the manual provided in the kit.

3.2.2 Determination of protein profile

(a) Sample preparation

Serum from selected patients (n=14) was treated using ProteoMiner™ Protein Enrichment Beads (Bio-Rad, USA) to decrease the levels of high-abundance protein and capturing low-abundance protein, thus leading to an enrichment of medium- and low-abundance protein. Each bead features a different hexapeptide ligand with affinity for specific proteins in a sample. When a complex protein sample is incubated with a bead library, protein components find their binding partners. Excess high-abundance protein will not be captured on binding sites and will be depleted once the beads are washed. In contrast, low-abundance proteins will be concentrated on their specific affinity ligand. In this way, low-abundance proteins are enriched relative to the high-abundance proteins in the sample. No fraction is discarded in this approach and proteins that might bind to high-abundance protein like albumin are retained. All the procedures were performed following the protocol provided in the kit; with the exception that only 500 µl of serum was used instead of 1 ml. In terms of 2D-GE run, each sample was run in duplicate.

(b) Protein quantification

Protein quantification was performed by using GE Healthcare Life Sciences 2-D Quant Kit. This kit was designed for accurate determination of protein concentration which is critical for high resolution electrophoresis techniques such as 2D-GE. All the procedures were performed following the manual

provided in the kit. Different concentrations of bovine serum albumin (BSA) were used to derive a standard curve which was then used to quantify the protein concentration in samples based on optical density.

The absorbance of each sample and standard were read at 490 nm using water as the reference. A standard curve was generated by plotting the absorbance of the standards against the quantity of protein. This standard curve was used to determine the protein concentration of the samples. Figure 4 shows a representative standard curve used in the experiment.

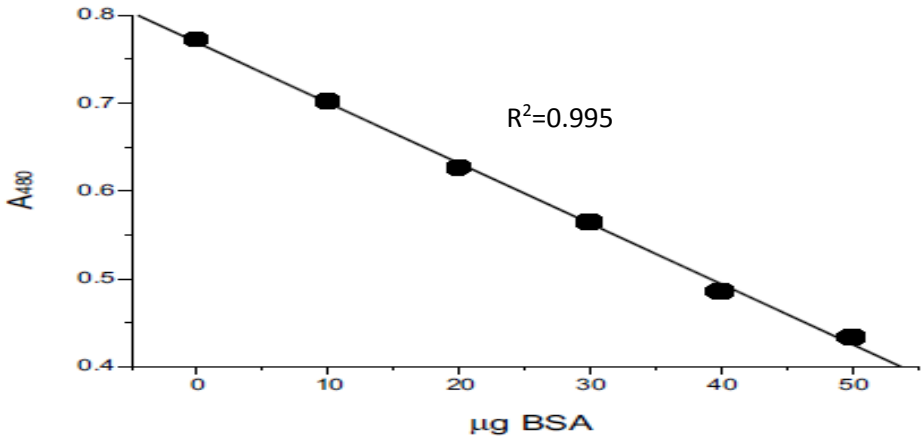


Figure 4: Standard curve used for protein quantification

(c) Sample clean-up

The kit used in this experiment was GE Healthcare Life Sciences 2-D Clean-Up Kit. The procedure conducted was based on the manual provided with the kit. The procedure was based on larger sample of more than 100 µg protein.

Briefly, precipitant (provided in the kit) was added into the samples and vortexed before leaving on ice for 15 min. Then, a co-precipitant was added to the mix before centrifuging at 8 000 x g for 10 min. The pellet was washed with co-precipitant and re-suspended in sterile Milli-Q water. Then, 1 ml of wash buffer and 5 µl of wash additives were added into the suspension before incubating the suspension at -20 °C for 30 min. The tube was vortexed for 20-30 sec once every 10 min during the incubation period. Following that, the tube was centrifuged at 8000 x g for 10 min before re-suspending the pellet in 250 µl of sample rehydration solution.

(d) Rehydration of IEF sample loading

In this study, IPG strips pH 3-10 of 11 cm length (GE Healthcare, Sweden) was used. Two hundred and fifty µg of protein was used from selected samples that had been treated with ProteoMiner™ Protein Enrichment Kits (Bio-Rad, USA).

The sample that had undergone clean-up (250 µg of protein) was pipette onto a reswelling tray. The IPG strip was removed from the pouch and the protective film was discarded using clean forceps. The strip was carefully placed into the reswelling tray with gel facing the sample to remove any air bubbles trapped

underneath the strip. Mineral oil (1.5 ml) was pipette on top of the strip to avoid rehydration. The strip was left in the reswelling tray for overnight (15 hrs) at room temperature to let the samples to be absorbed into the gel.

(e) 1-Dimension (1-D) separation

After the overnight incubation, the strips were carefully removed from the reswelling tray, onto a Whatman filter paper by using a clean forcep. This step was performed to remove the excess mineral oil. A manifold was placed on the Ettan IPGphor3 platform (GE Healthcare, Sweden) before it was filled with the mineral oil. The strips were carefully transferred onto a manifold. The strips were positioned under the cover fluid, with the gel facing upwards in the tray with the anodic (+, pointed) end of the IPG strip pointing at the anode of the Ettan IPGphor 3. On each end of the gel, a damp wick was placed, overlapping the end of the gel on the IPG strip. The electrode were positioned and assembled on top of all the wicks, with the electrode in contact with the wicks.

The 1-D separation was performed at four cycles; the first cycle was run at 500 volt (V) for 1 hr at 0.5 kVh, with the requirement of step and hold. The second cycle at 1 000 V for 1 hr at 0.8 kVh, with the requirement of gradient. The third cycle was performed at 6 000 V for 2 hrs at 7.0 kVh with the same requirement as the second cycle. The last cycle requires 6 000 V for 40 min at 3.7 kVh, with the requirement of step and hold. After the cycles were completed, the strips were transferred to a clean glass tube, capped and stored at -80 °C until used.

(f) Two-dimension gel electrophoresis (2D-GE)

Two-dimension gel electrophoresis was carried out in vertical gel electrophoresis system (SE 600 Ruby, GE Healthcare, Germany). The strips were equilibrated in 5 ml of SDS equilibration buffer with 0.05 g dithiothreitol (DTT) in solution for 15 min. The solution was then discarded and replaced with 5 ml SDS equilibration buffer that was added with 0.125 g of iodoacetamide (IAA) and incubated for 15 min. After the equilibration, the strips were washed with SDS running buffer to remove all the excess equilibration solution. By using forceps, the strips were carefully placed on top of the second dimension gel that had been casted. The gels were placed in the 2D-GE running tank which was then filled with running buffer. The 2D-GE was carried at two different successive steps. The first step was performed at 50 V, 160 mA, and 100 W for 30 min. This is followed by the second step which is 600V, 160 mA and 100 W for 4 hrs. This setting was used when 4 samples were run together.

(g) PlusOne™ silver staining

After completion of the 2D-GE run, the gels were removed carefully from the glass plate and placed into a suitable stainless steel container. The gels were soaked overnight with fixing solution (40 % ethanol, 10 % acetic acid). The next day, the fixing solution was removed and 250 ml of sensitizing solution was added into the tray and left shaking on a rotary shaker for 1 hr. The solution was discarded and the gel was washed with Milli-Q water four times for 5 min. Then 250 ml of silver solution was added onto the gel and left for 30 min on the

rotary shaker. The solution was discarded and the gels were washed four times for one minute each in sterile Milli-Q water. Following that, 250 ml of freshly prepared developing solution was poured onto gel and rotated for 2-4 min or until spots begun to form. Then, the gels were immediately transferred into sterile Milli-Q water for 2-3 min before transferring them into 250 ml of stop solution for 1 hr. Then, the gel was kept into sterile distilled water for storage up to 1 week or was kept into 5 % acetic acid for longer period.

(h) Image analyser

The proteomic images for all gel were acquired using GS-800 Calibrated Densitometer (Bio-Rad, Germany) and analysed using PD Quest 2-D Analysis Software (Bio-Rad, Germany). Spots that were consistently and significantly different were selected and subjected to trypsin digestion.

(i) In-gel trypsin digestion

Silver stained gel spots were excised, destained with 15 mM potassium ferricyanide in 50 mM sodium thiosulphate, 10 mM DTT in 100 mM ammonium bicarbonate, alkylated with 55 mM of IAA in 100 mM ammonium bicarbonate in the dark, dehydrated with acetonitrile (ACN) and reswollen in 6 ng/µl trypsin in 50 mM ammonium bicarbonate at 37^{o}C overnight. After overnight digestion, the hydrolysates were transferred to 0.5 ml tubes and tryptic peptides were further extracted from the gel pieces using 50 µl of water/ACN

solution with vigorous vortexing for 15 min. The extraction process was repeated again using 100 % ACN. The final hydrolysate supernatants were concentrated by vacuum centrifugation at low speed and stored at -20 °C until use.

(j) Protein identification by mass spectrometry

The digested tryptic were reconstituted in 0.1% of formic acid in distilled water. Then, the digested tryptic were aspirate subsequently in solution of 50% ACN, 0.1% formic acid and 0.1% formic acid in 50% ACN, respectively. The samples were eluted on MALDI plates. Raw data for protein identification was obtained on the MALDI TOF/TOF (ABI 4800 Plus) at the University of Malaya Centre for Proteomics Research, Medical Biotechnology Laboratory, Medical Faculty, University of Malaya.

(k) ELISA for protein verification

The kit used in this experiment was NovaTeinBio Protein ELISA Kit. The procedure conducted was based on the manual provided with the kit. All D1, D2 and D3 samples were used in this test.

This kit uses a double-antibody sandwich ELISA to analyze the level of the wanted-protein in samples. The diluted standards, diluted samples and blanks

were added to the wells that have been pre-coated with the protein antibody to capture available analyte in solution. Then, HRP-conjugated was added to the wells (except the blank well) to bind the captured analyte. The wells were mixed gently. Then, the wells were incubated at 37 °C for 1 hr and washed to remove unbound substance and unbound antibody-HRP. Finally, Chromogen Substrate A and B were added to each well, mixed and incubated for development of blue colour for 15 min at 37 °C. Finally, a stop solution was added in each well and the colour reaction turns the mixture to yellow. The yellow color intensity proportionally correlates to the concentration of the protein in samples. The optical density of each well was measured at 450 nm within 15 min after the addition of stop solution.

CHAPTER 4

RESULTS

CHAPTER FOUR: RESULTS

4.1 Determination of dengue status

4.1.1 Dengue NS1 Ag-Ab Combo Kit

Patients that were admitted to HTAR due to dengue fever were recruited in this study (n=100). The dengue status of each patient was immediately determined by using the SD BIOLINE™ Dengue Duo rapid test kit. This test kid is designed for the detection of both dengue virus NS1 antigen and differential IgM/IgG antibodies to dengue virus in human whole blood, serum or plasma. Table 3 list the results of NS1 Ag and IgG/IgM for the 100 patients selected for the study. The subjects were categorised into two different groups; Intervention (I) and Control (C). 49% of all dengue patients recruited tested positive for SD Duo NS1 Ag; and 83% patients tested positive for SD Duo IgM/IgG.

4.1.2 Dengue virus PCR for determination of serotype

In order to determine the value of the Tm of each serotype of dengue virus, the mean and the standard deviation of Tm were calculated for each serotype. Figure 5 and Figure 6 show the melting curves from selected patients from both groups. The melting curve analysis from group I (Fig 5) showed that the mean of Tm DENV-1 was 85.1 °C, DENV-2 was 82.5 °C, DENV-3 was 85.9 °C and DENV-4 was 83.1 °C. In group C (Fig 6), the mean of Tm of DENV-1 was 85.0 °C, DENV-2 was 82.5 °C, DENV-3 was 85.8 °C and DENV-4 was 83.4 °C. No peak was seen for the negative control as there was no amplification in the

Table 3: Results of rapid test for both Intervention (I) and Control (C) group

Rapid Test	**Intervention (I) (%)**		**Control (C) (%)**	
NS1 / **IgM/IgG**	Positive	Negative	Positive	Negative
Both Positive	40	52	28	46
IgM Positive	6	0	14	0
IgG Positive	0	0	2	4
Both Negative	2	0	6	0

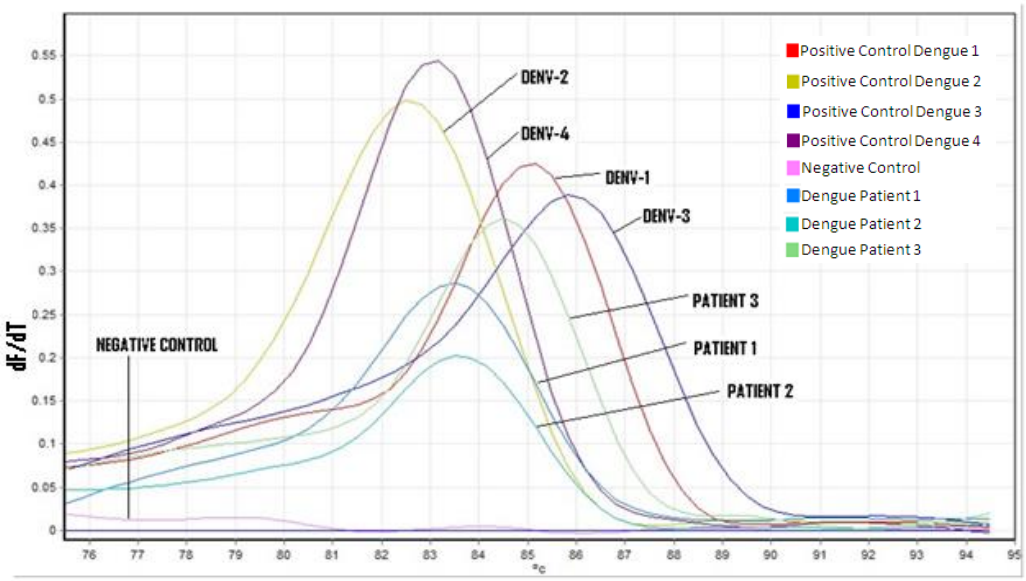


Figure 5: The melting curve analysis of three selected patients in Intervention (I) group. Melting curves of dengue virus prototypes were used as reference to determine the serotype of patients.

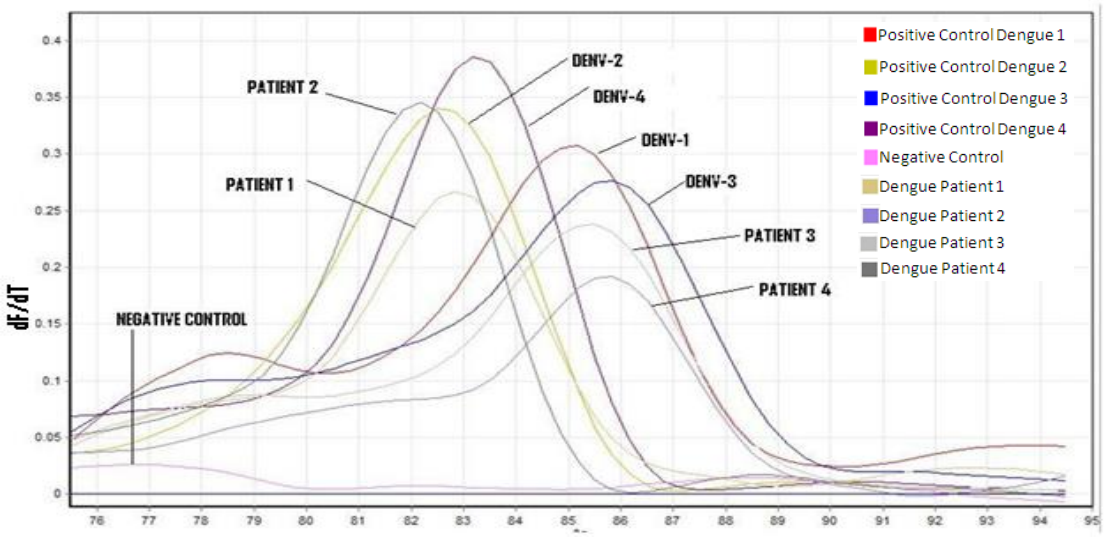


Figure 6: The melting curve analysis of four selected in Control (C) patients. Melting curves of dengue virus prototypes were used as reference to determine the serotype of patients.

PCR reaction. The four strains of dengue viruses serotype that were used as positive control were prototype identified as DENV-1 from Hawaii, DENV-2 from New Guinea C, DENV-3 from H87 and DENV-4 from H241 (Yong *et al*., 2007).

All four serotypes were documented in these two groups. The most dominant serotype in group I was DENV-1 (36 %), followed by DENV-2 (22 %), DENV-4 (22 %) and DENV-3 (20 %). In group C, the most dominant serotype was also DENV-1 with 32 %. This was followed by DENV-2 (14 %), DENV-3 (14 %) and DENV-4 (6 %). Another 34% were unable to be detected by PCR. The results are tabulated in Table 4.

4.1.3 IgM Enzyme-Linked Immunosorbent Assay (IgM-ELISA)

The Panbio Dengue IgM Capture ELISA was used to determine the status of infection in patients. If the IgM could be detected in the serum of a patient, it indicates that the patient was having a primary infection. The immune response during the primary dengue infection is characterised by a rise of IgM antibodies by the 3rd day of onset of symptoms and remain in the body for about 3 months. Table 5 shows the results of IgM in both groups whereby patients in group I that are positive for IgM was 92 % while in group C was 84 %.

Table 4: Distribution of serotypes for positive samples in Intervention (I) group and Control (C) group.

Serotype	Positive samples (%)	
	Intervention (I)	Control (C)
DENV-1	36	32
DENV-2	22	14
DENV-3	20	14
DENV-4	22	6
None	0	34

Table 5: Distribution of positive IgM in samples from group I and C.

ELISA IgM	Intervention (I) (%)	Control (C) (%)
Positive	92	84
Negative	8	16

4.2 2D-GE experiments using serum from healthy individuals and patients from I and C group

About 250 µg of protein from serum treated with ProteoMiner™ Protein Enrichment Kits (Bio-Rad, USA) was sufficient to derive 2D-GE profiles. Before the 2D-GE experiments were carried out, the total protein content in all these samples were quantified. This was done upon treatment of the samples with lysis buffer and subsequently all inhibitory components were removed by 2D-GE clean up. The volume needed to get 250 µg of proteins after depletion using ProteoMiner™ was in the range of 37-60 µl. The gels were stained by using PlusOne® Silver Staining to get more and clearer spots. This kit is mass-spectrometry compatible. The gels were then scanned by using GS-800 Calibrated Densitometer (Bio-Rad, Germany).

4.2.1 2D-GE profile of serum from healthy individuals

2D-GE using sera of healthy individuals were performed as a part of the optimization of the procedure. The profile obtained was compared with the profiles developed by other research groups utilizing the ProteoMiner™ kit (Fekkar *et al.*, 2012). A representative 2D-GE profile for sera of a healthy individual is shown in Figure 7. The gel was resolved with 1043 protein spots with molecular masses ranging from as low as 8 kDa to as high as 195 kDa.

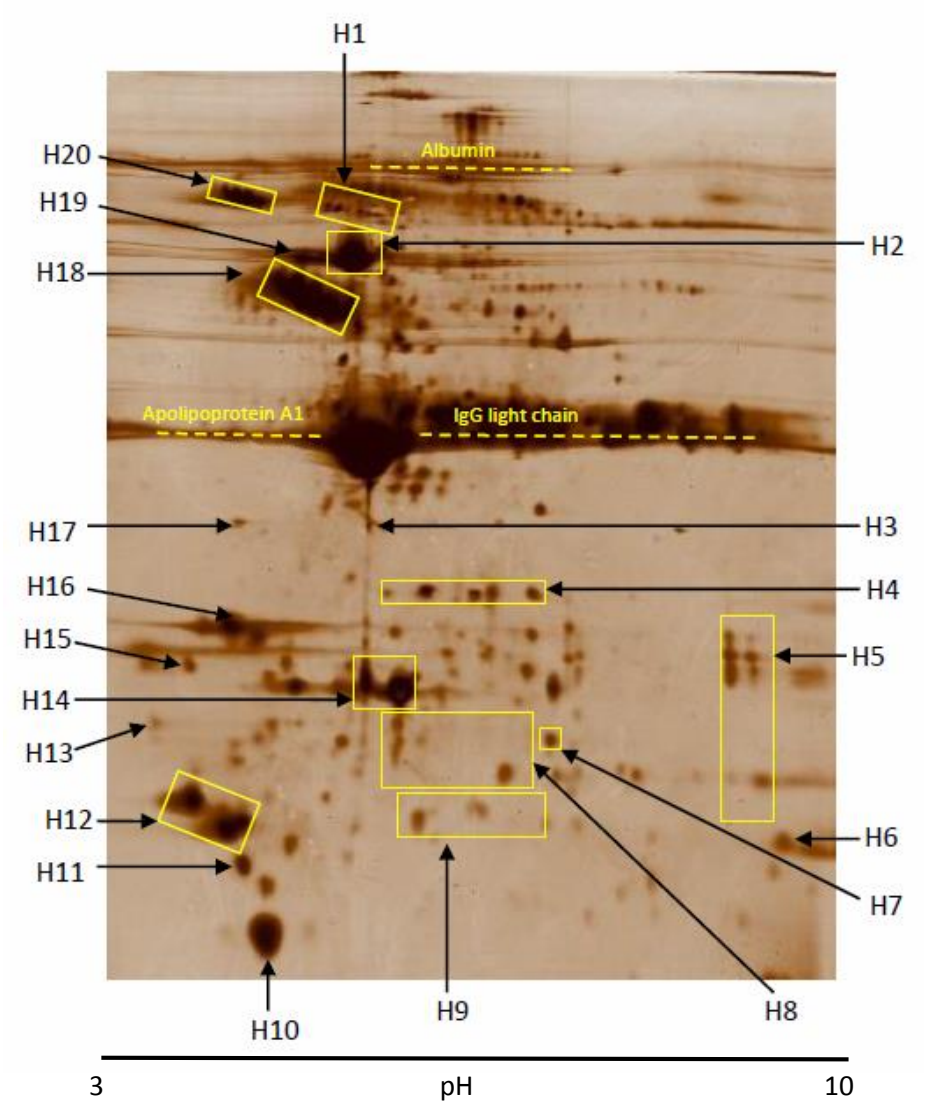


Figure 7: Representative 2D-GE profile of proteins in sera of healthy individuals.

The gel was resolved with 1043 protein spots with molecular masses ranging from as low as 8 kDa to as high as 195 kDa. Spots that were identified by using MS analysis is boxed and labeled H1 to H20 and their identities are shown in Table 6.

The spots that were obtained were sent for MS analysis. A total of 22 proteins were identified in the profile picture of the healthy individuals and they were α2-HS-glycoprotein (AHSG), Apolipoprotein groups (A-I, A-II, A-IV, C-II, C-III and J), component protein group (4A and C-III), haemoglobin, haptoglobin α1 chain, haptoglobin α2 chain, prothrombin, retinol binding protein 4, SAA human, transthyretin, vitamin-D binding protein, and vitronectin. Table 6 shows the proteins that were identified from the healthy individual's protein profile.

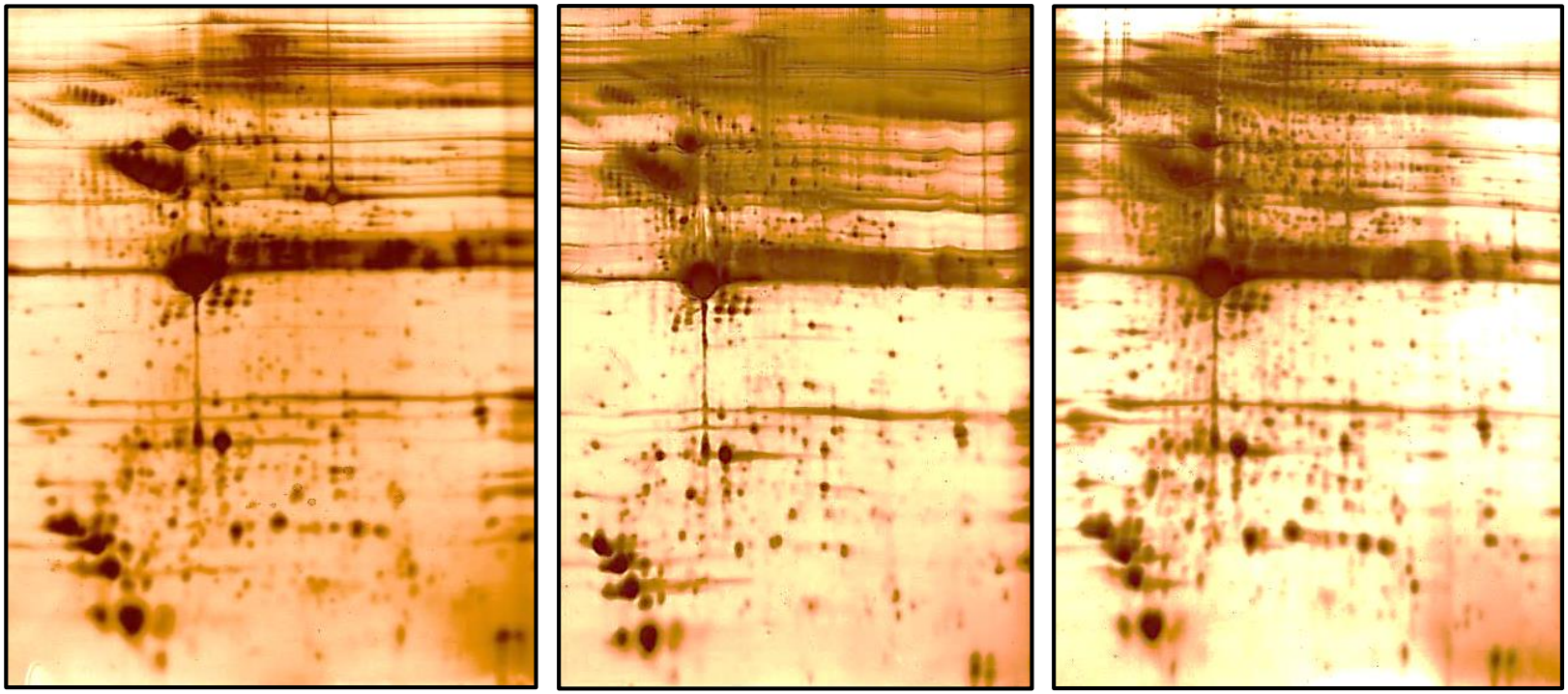
4.2.2 2D-GE profile of patients of I and C group

2D-GE was performed for the I (n=7) and C (n=7) group for Day 1, 2 and 3. Initially, the profiles that were obtained were compared visually in order to identify differentially expressed spots. 2D-GE profiles for I and C group were shown in Figure 8 and 9, respectively.

Visual comparison between the profiles of serum proteins obtained in both I and C groups shows that more spots were present at the lower part of the protein profile in group I compared to group C. In addition, more protein spots were observed at the upper part of the protein profile in D3 compared to D2 and D1 for both I and C groups. Comparisons of the serum protein profiles were done within the group in order to observe the effect of CPL juice and to observe the pattern of protein regulations in the treated (I) and non-treated (C) groups.

Table 6: List of proteins that were identified in the 2D-GE profile of healthy individuals (as shown in Figure 7)

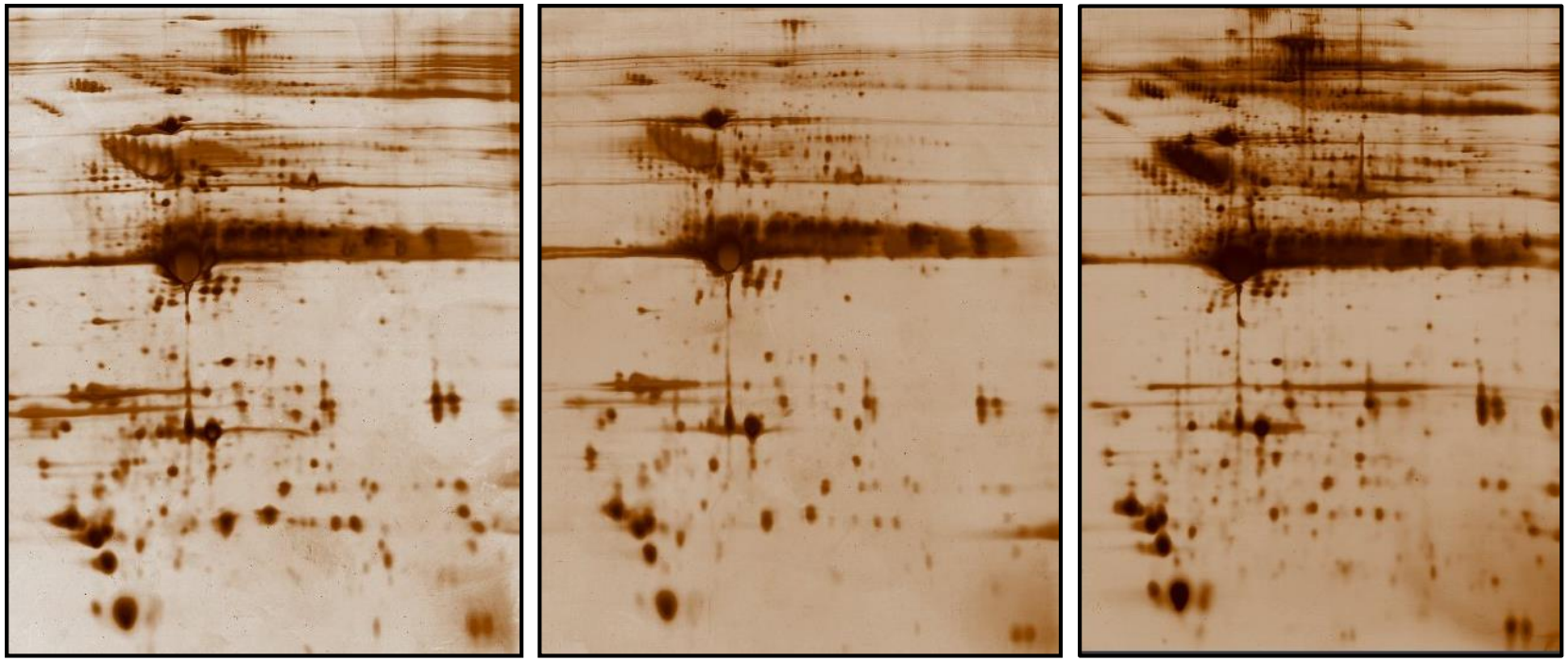
Spot No	Protein ID	Nominal mass (Mr)	Calculated p*I*
H1	Vitamin D binding protein	52929	5.4
H2	Apolipoprotein A-IV	45371	5.28
H3	Retinol Binding Protein (RBP) 4	23271	5.77
H4	Haptoglobin α2 chain	45861	6.13
H5	Serum Amyloid A Human	14855	9.04
H6	Complement component C3	188569	6.02
H7	Haemoglobin subunit beta	16102	6.75
H8	Haptoglobin α1 chain	45861	6.13
H9	Serum Amyloid A Human	13611	6.28
H10	Apolipoprotein A-II	11282	6.26
H11	Apolipoprotein C2	8141	4.86
H12	Apolipoprotein C3	12864	7.9
H13	Vitronectin	54271	5.55
H14	Transthyretin	20300	5.16
H15	Prothrombin	69992	5.64
H16	Complement component 4A	194351	6.59
H17			
H18	Apolipoprotein J (clusterin)	52461	5.89
H19	Complement component C3	187030	6.02
H20	alpha-2-HS-glycoprotein (AHSG)	39300	5.43
	Albumin	Reference : Sriyam *et al*., 2007	
	Apolipoprotein A-I	30759	5.56
	IgG light chain	24300	



ID1 **ID2** **ID3**

Figure 8: 2D-GE serum profile of group I patients for pre-treatment (ID1), post-treatment (ID2 and ID3).

The profiles were acquired by GS-800 scanner (Bio-Rad, Germany). Total protein load was 250 µg per each 11 cm IPG strips pH 3-10. Isoelectric focusing was performed for a total of 4 hrs and 40 min, followed by second dimension electrophoresis. Gels were stained with silver staining.



CD1 **CD2** **CD3**

Figure 9: 2D-GE serum profile of group C patients (CD1, CD2 and CD3).

The profiles were acquired by GS-800 scanner (Bio-Rad, Germany). Total protein load was 250 µg per each 11 cm IPG strips pH 3-10. Isoelectric focusing was performed for a total of 4 hrs and 40 min, followed by second dimension electrophoresis. Gels were stained with silver staining.

4.2.3 Image analysis of group I and C

The protein profiles from both the I and C groups were then compared and the mean of standard spot (SSP) number for each protein spots were analysed by using PD Quest 2-D Analysis Software (Bio-Rad, Germany). The SSP number represents the spot's quantity of 2D-GE-resolved serum proteins. Gel images were normalized and volume differences were statistically calculated. The differentially expressed proteins that have a minimum of two-fold regulation between D1 and D2 and a minimum of one-fold regulation between D2 and D3 were identified. Spots that were consistently and significantly different were selected and subjected to trypsin digestion, and were further analysed by mass-spectrometry. The identified proteins that were differentially expressed are shown in Figure 10. The lists of the proteins identified by mass spectrometry are shown in Table 7. The proteins were vitamin-D binding protein (VDBP), alpha-2-HS-glycoprotein (AHSG), complement component C3 (Comp C3), clusterin (CLU), vitronectin (VNR) and apolipoprotein C3 (APO C3).

Statistical analysis was performed using the Paired T-Test in GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The t-test analysis were done by comparing the mean of SSP number of D1 against D2 and D2 against D3 in each I and C group. A p-value of less than 0.05 was considered significant.

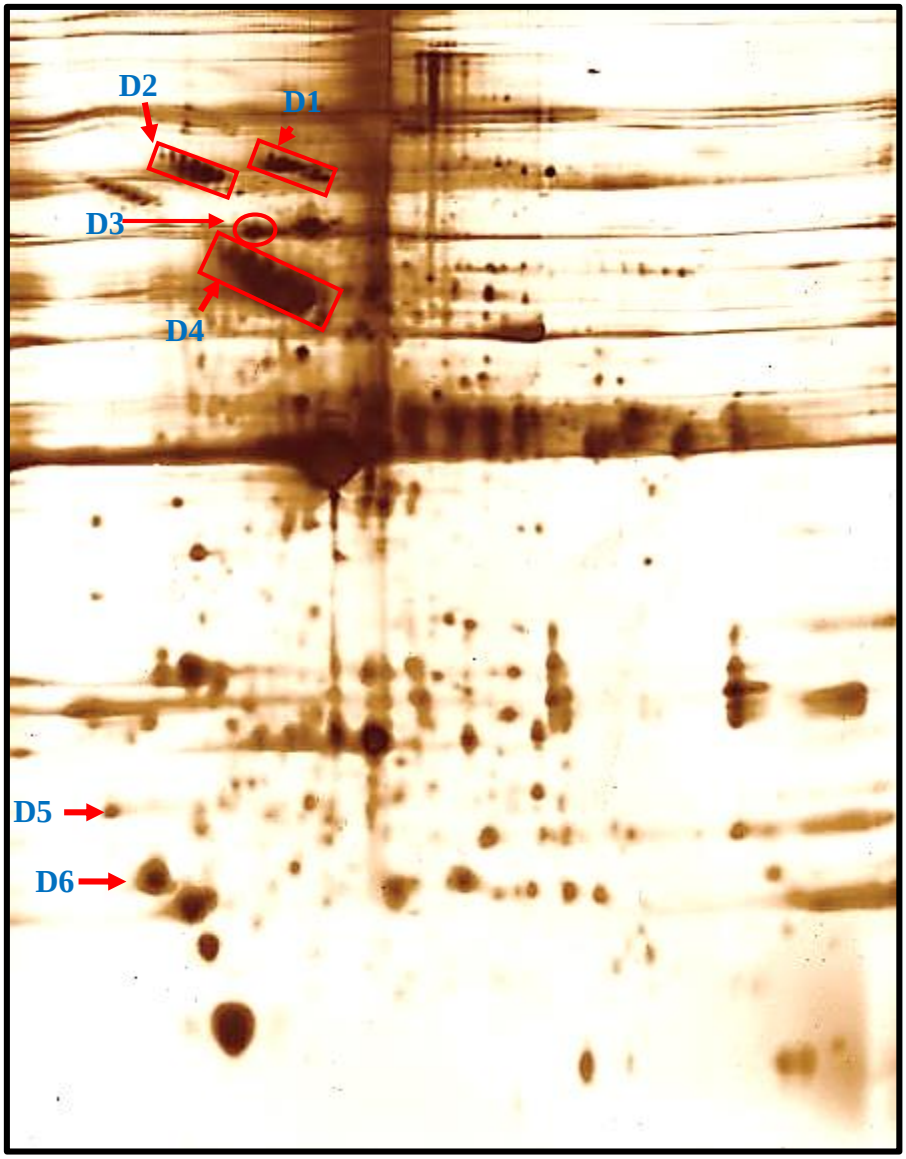


Figure 10: A representative gel from group ID2. Image analysis was performed to detect differentially expressed protein that were significantly detected within the I and C group. Spots labeled D1-D6 are identified by MS analysis and their identities are shown in Table 7

Table 7: Mass spectrometric identification of differentially expressed protein between Day 1, Day 2 and Day 3 in Intervention and Control group

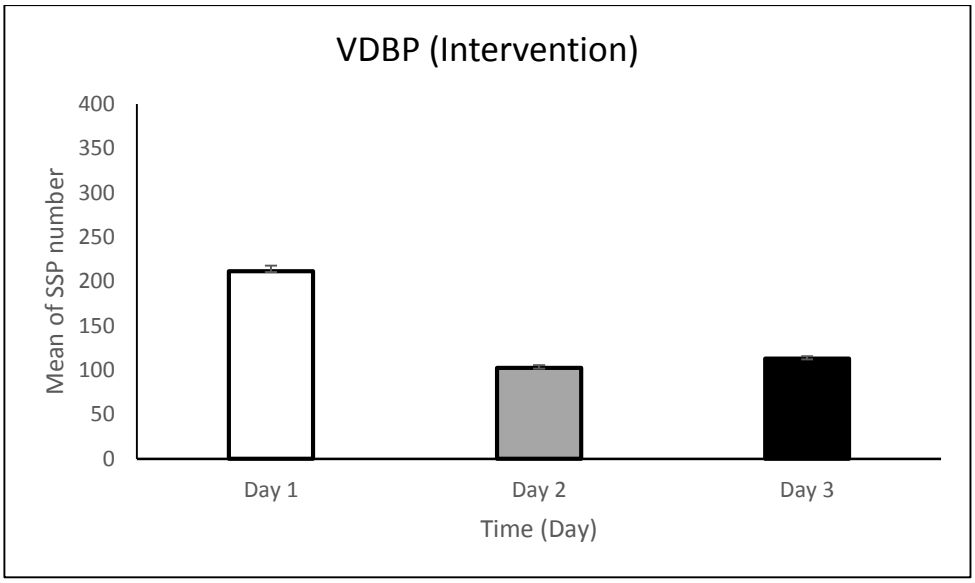
Spot No	**Protein ID**	**Sequence coverage (%)**	**Nominal mass (M_r)**	**Calculated p*I***
D1	Vitamin D-binding protein	32	52929	5.40
D2	Alpha-2-HS-glycoprotein	29	39300	5.43
D3	Complement C3	11	187030	6.02
D4	Clusterin	29	52461	5.89
D5	Vitronectin	5	54271	5.55
D6	Apolipoprotein C3	47	12864	7.9

The comparison of protein profiles between D1 and D2 in group I showed that two proteins were up-regulated and four proteins were down-regulated. The up-regulated proteins were Comp C3 (2.01 fold, $p<0.0001$) and APO C3 (2.02 fold, $p<0.0001$). The down-regulated proteins were VDBP (2.07 fold, $p<0.001$), AHSG (2.09 fold, $p<0.0001$), CLU (2.01 fold, $p<0.0001$) and VNR (2.04 fold, $p<0.0001$). However, three proteins were found to be up-regulated and three proteins were found to be down-regulated when the comparison was done between D2 and D3 in group I. The up-regulated proteins were VDBP (1.10 fold, $p<0.0001$), AHSG (1.45 fold, $p<0.0001$) and CLU (1.36 fold, $p<0.0001$), while the down-regulated proteins were Comp C3 (1.15 fold, $p<0.0001$), VNR (1.80 fold, $p<0.0001$) and APO C3 (1.25 fold, $p<0.0001$).

In group C, the comparison of protein profiles between D1 and D2 showed that four proteins were found to be up-regulated while another two proteins were found to be down-regulated. The up-regulated proteins were VDBP (2.03 fold, $p<0.0001$), CLU (1.10 fold, $p<0.0001$), VNR (2.52 fold, $p<0.0001$) and APO C3 (1.32 fold, $p<0.0001$). The down-regulated proteins were AHSG (1.24 fold, $p=0.0005$) and Comp C3 (1.35 fold, $p=0.0001$). The same proteins showed the similar regulation when the protein profiles of group C were compared between D2 and D3. VDBP (1.55 fold, $p<0.0001$), CLU (1.30 fold, $p<0.0001$), VNR (1.22 fold, $p<0.0001$) and APO C3 (1.20 fold, $p<0.0001$) were up-regulated, while AHSG (1.33 fold, $p=0.0214$) and Comp C3 (1.51 fold, $p=0.0003$) were down-regulated.

All of the identified differentially expressed proteins showed significant value of difference between D1 against D2 and D2 against D3. Figure 11, 12, 13, 14, 15 and 16 show the histogram that was plotted for the mean of SSP number for each VDBP, AHSG, CLU, Comp C3, VNR and APO C3 proteins in Intervention and Control group, respectively. Table 8 shows the relative expression of serum proteins analyzed by PD Quest 2-D Analysis Software (Bio-Rad, Germany). The data for the mean of SSP number for each group can be referred in the Appendix I.

(a)



(b)

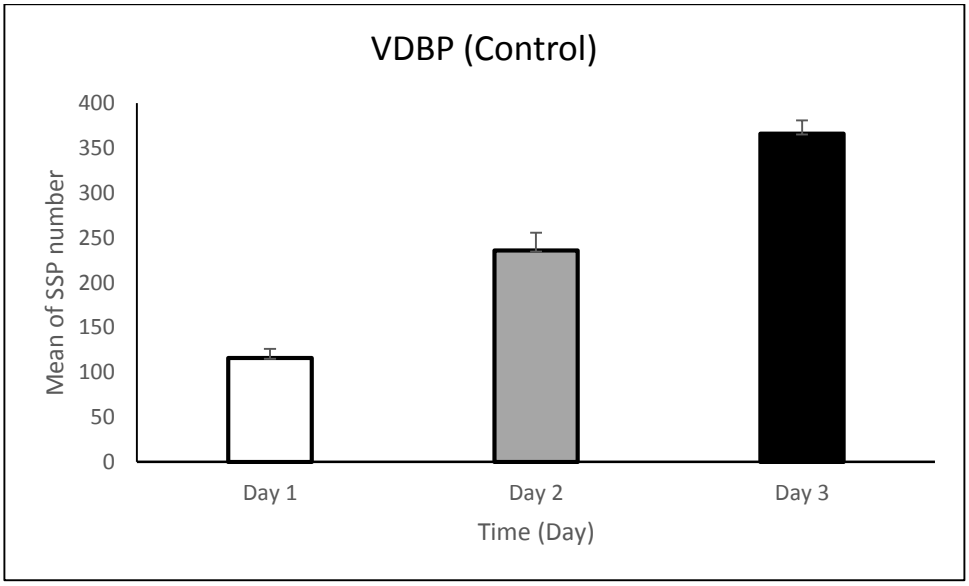
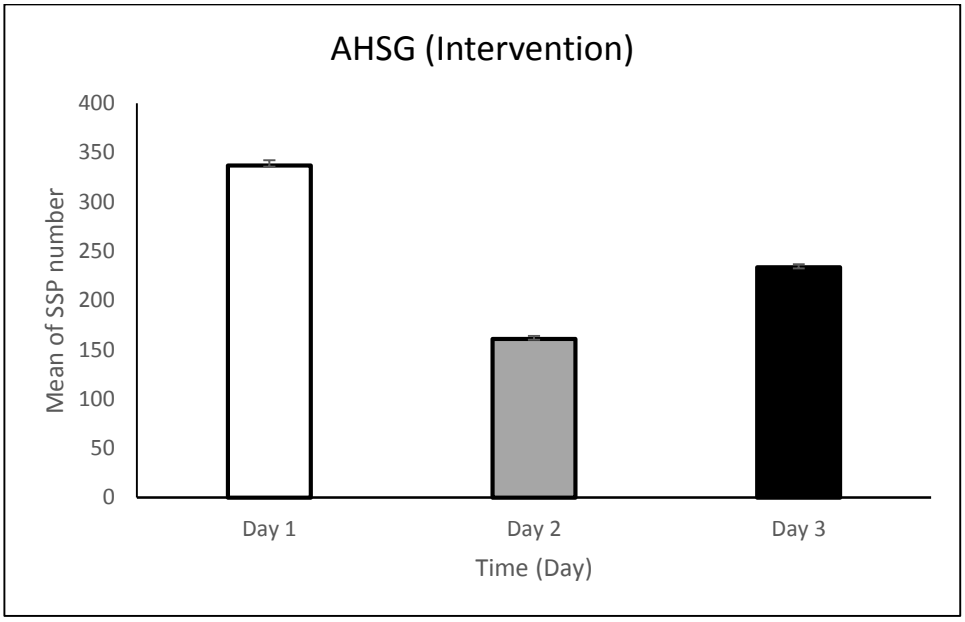


Figure 11: Mean of SSP number of VDBP in (a) Intervention and (b) Control group

(a)



(b)

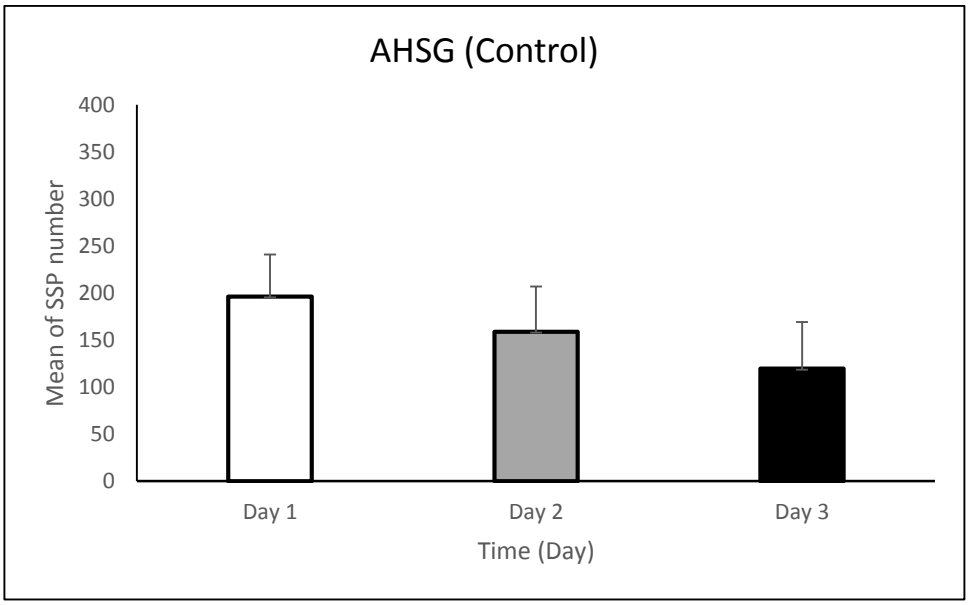
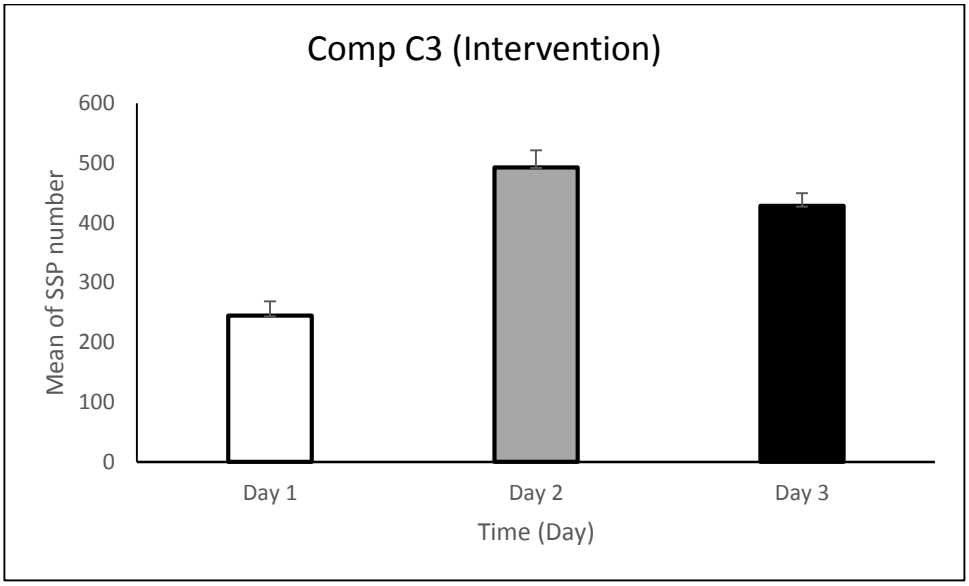


Figure 12: Mean of SSP number of AHSG in (a) Intervention and (b) Control group

(a)



(b)

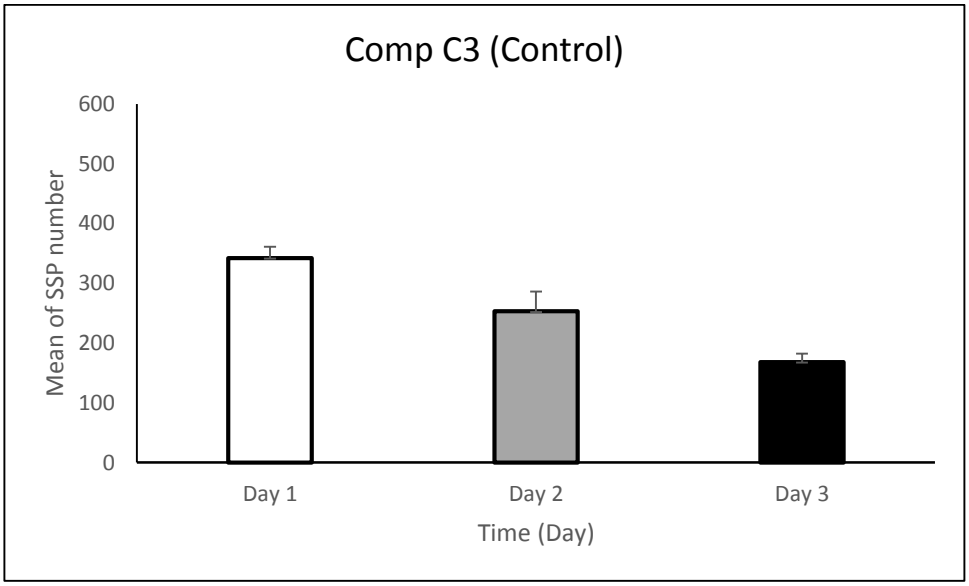
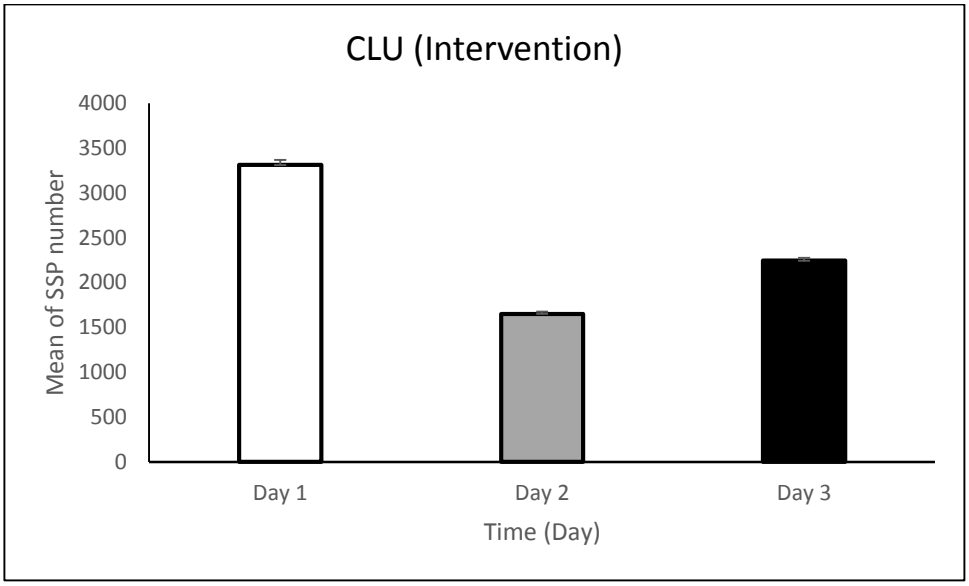


Figure 13: Mean of SSP number of Comp C3 in (a) Intervention and (b) Control group

(a)



(b)

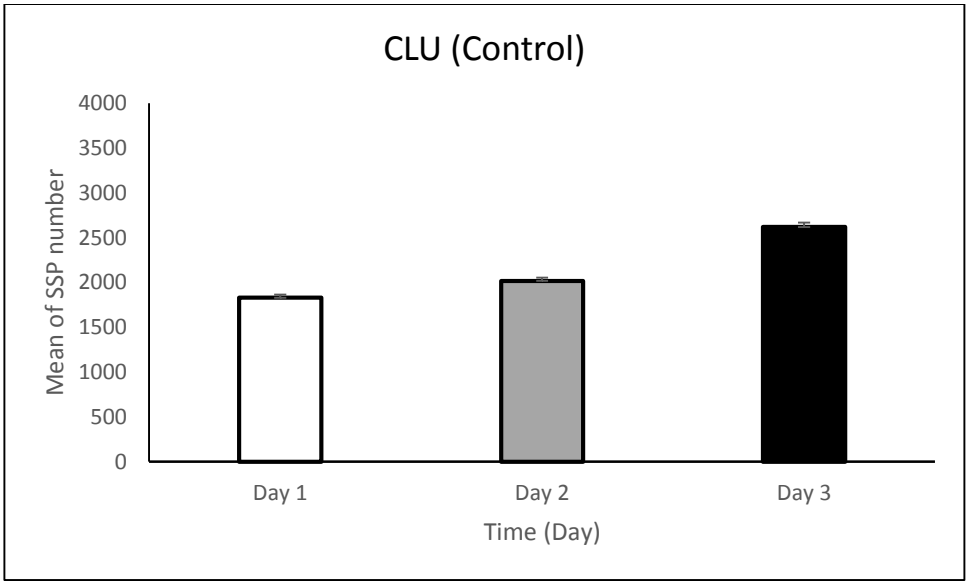
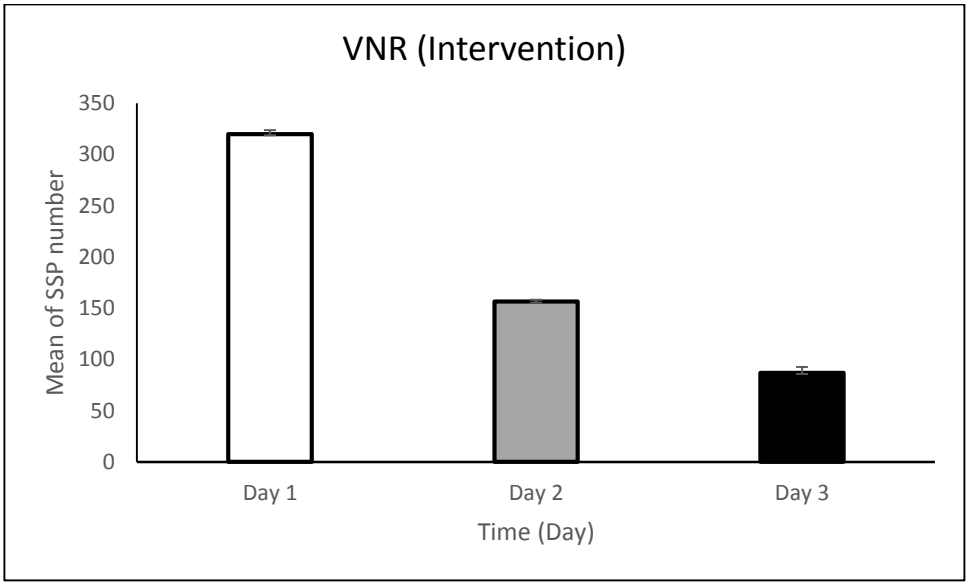


Figure 14: Mean of SSP number of CLU in (a) Intervention and (b) Control group

(a)



(b)

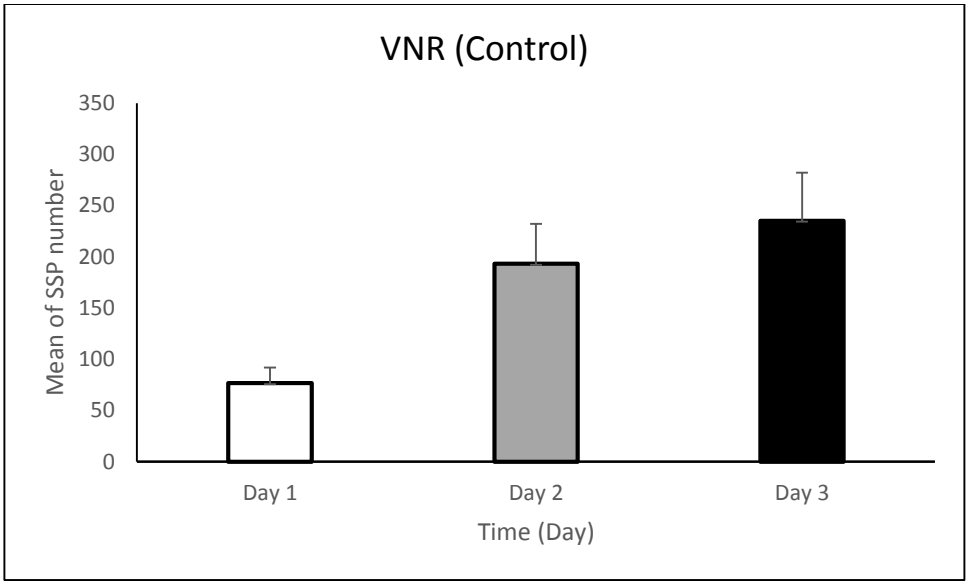
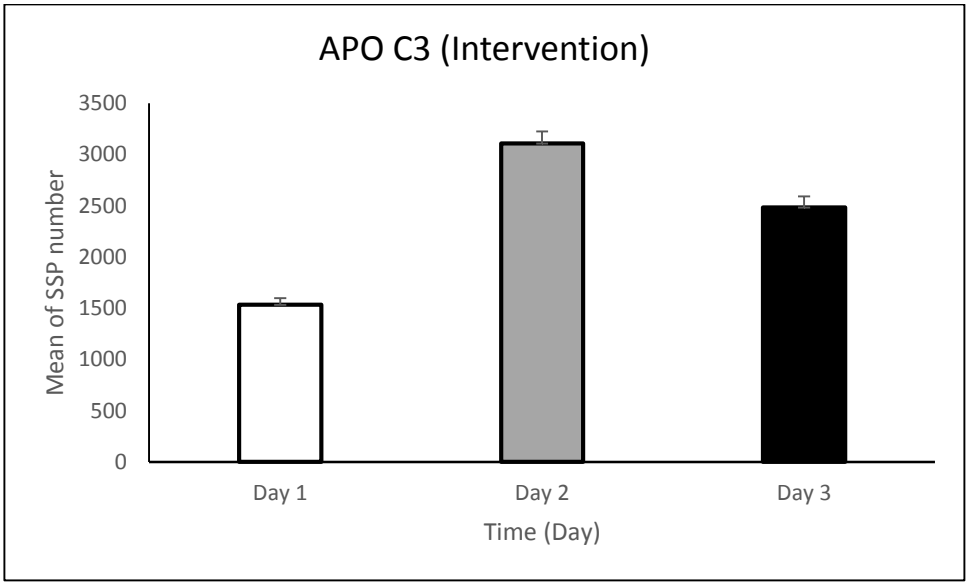


Figure 15: Mean of SSP number of VNR in (a) Intervention and (b) Control group

(a)



(b)

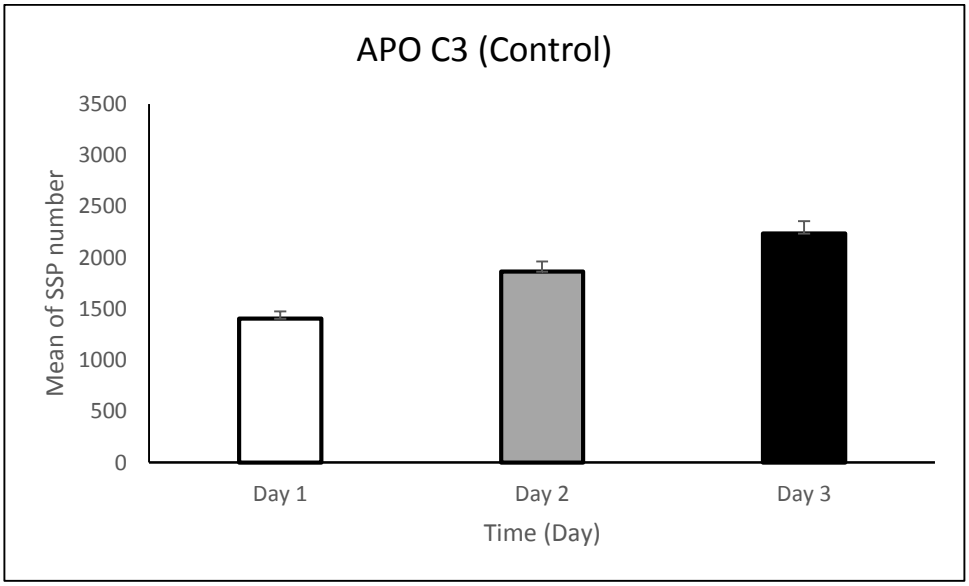


Figure 16: Mean of SSP number of APO C3 in (a) Intervention and (b) Control group

Table 8: Relative expression of serum proteins analyzed by 2D-GE

Serum Proteins	Intervention (Fold changes relative to the previous day)		Control (Fold changes relative to the previous day)	
	Day 2	Day 3	Day 2	Day 3
VDBP	-2.07 (p< 0.0001)	+1.10 (p< 0.0001)	+2.03 (p< 0.0001)	+1.55 (p< 0.0001)
AHSG	-2.09 (p< 0.0001)	+1.45 (p< 0.0001)	-1.24 (p=0.0005)	-1.33(p=0.0214)
Comp C3	+2.01 (p< 0.0001)	-1.15 (p< 0.0001)	-1.35 (p= 0.0001)	-1.51 (p= 0.0003)
CLU	-2.01 (p< 0.0001)	+1.36 (p< 0.0001)	+1.10 (p< 0.0001)	+1.30 (p< 0.0001)
VNR	-2.04 (p< 0.0001)	-1.80 (p< 0.0001)	+2.52 (p< 0.0001)	+1.22 (p< 0.0001)
APO C3	+2.02 (p< 0.0001)	-1.25 (p< 0.0001)	+1.32 (p< 0.0001)	+1.20 (p<0.0001)

+ Increase in expression; - decrease in expression

4.2.4 ELISA for protein verification

The proteins that were identified to be significantly different in both the I and C groups were verified by using ELISA from NovaTeinBio, USA. The proteins were VDBP, AHSG, Comp C3, VNR, CLU and APO C3. The assay was carried out for all samples (n=100) for each D1, D2 and D3 from both the I and C groups.

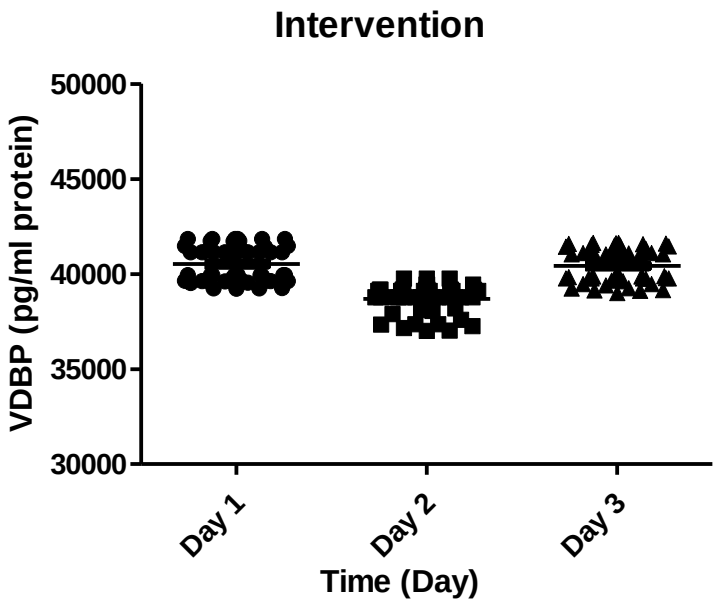
The ELISA assay that was performed for all six proteins showed a similar regulation with the findings of 2D-GE. All of the six proteins were regulated at a minimum of one-fold between D1 and D2 and also between D2 and D3 for both the I and C group. Paired T-Test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The t-test analysis were done by comparing the mean of protein concentration between D1 against D2 and D2 against D3 in each I and C group. A p-value of less than 0.05 was considered significant.

In group I, Comp C3 (1.59 fold, $p<0.0001$) and APO C3 (1.28 fold, $p<0.0001$) were found to be up-regulated while VDBP (1.05 fold, $p<0.0001$), AHSG (1.05 fold, $p<0.0001$), CLU (1.03 fold, $p<0.0001$) and VNR (1.35 fold, $p<0.0001$) were found to be down-regulated from D1 to D2. However, three proteins were found to be up-regulated and three proteins were found to be down-regulated when the comparison was done between D2 and D3. The up-regulated proteins were VDBP (1.04 fold, $p<0.0001$), AHSG (1.04 fold, $p<0.0001$) and CLU (1.02 fold, $p<0.0001$), while the down-regulated proteins were Comp C3 (1.31 fold, $p<0.0001$), VNR (1.38 fold, $p<0.0001$) and APO C3 (1.35 fold, $p<0.0001$).

In group C, four proteins were up-regulated and two proteins were down-regulated when comparison was done between D1 and D2. The up-regulated proteins were VDBP (1.05 fold, $p<0.0001$), CLU (1.02 fold, $p<0.0001$), VNR (1.22 fold, $p<0.0001$) and APO C3 (1.93 fold, $p<0.0001$). The down-regulated proteins were AHSG (1.03, $p<0.0001$) and Comp C3 (1.38 fold, $p<0.0001$). The same regulation pattern was observed in these proteins when the comparison was done between D2 and D3.

Figure 17, 18, 19, 20, 21 and 22 show the mean of protein concentration of VDBP, AHSG, Comp C3, CLU, VNR and APO C3 respectively, for both the I and C group. Table 9 shows the relative expression of the differentially expressed proteins for both groups.

(a)



(b)

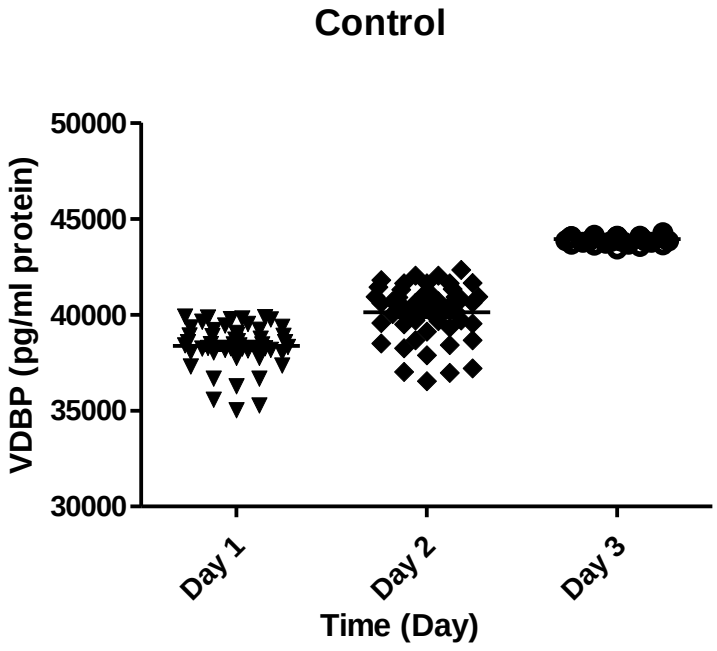
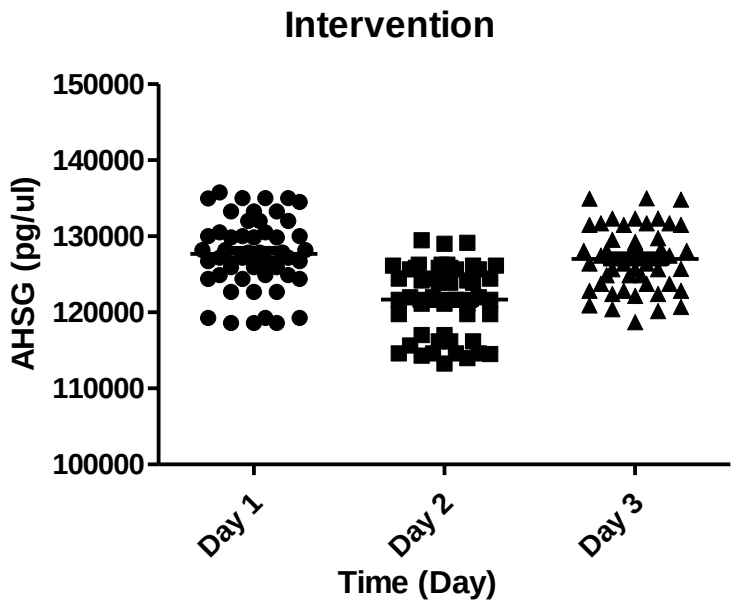


Figure 17: Mean of VDBP concentration in (a) Intervention and (b) Control group.

(a)



(b)

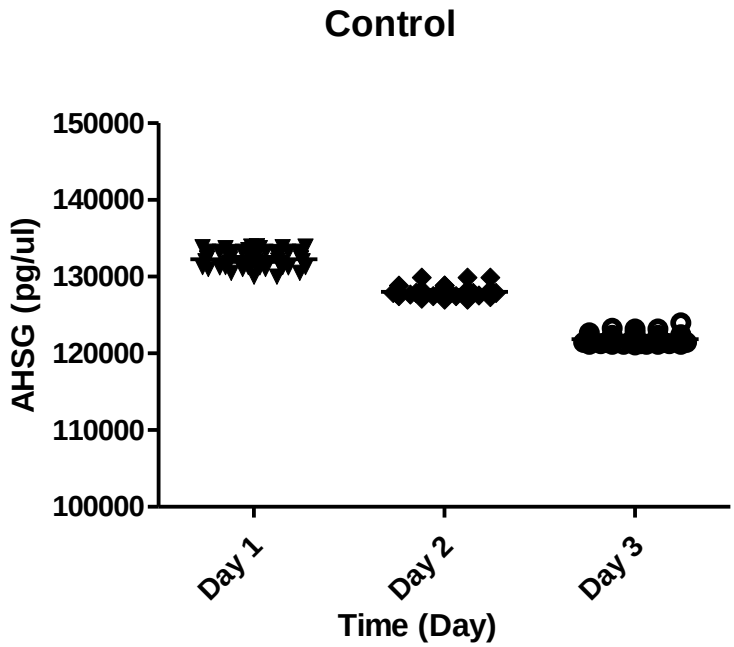
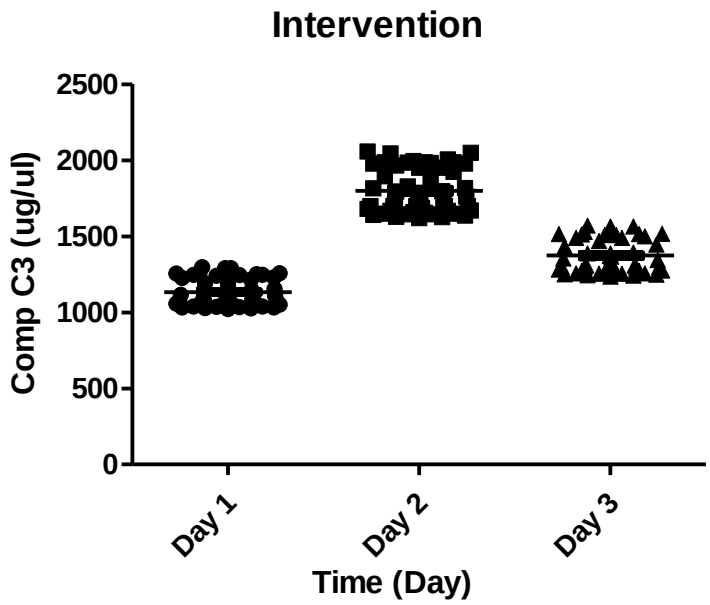


Figure 18: Mean of AHSG concentration in (a) Intervention and (b) Control group.

(a)



(b)

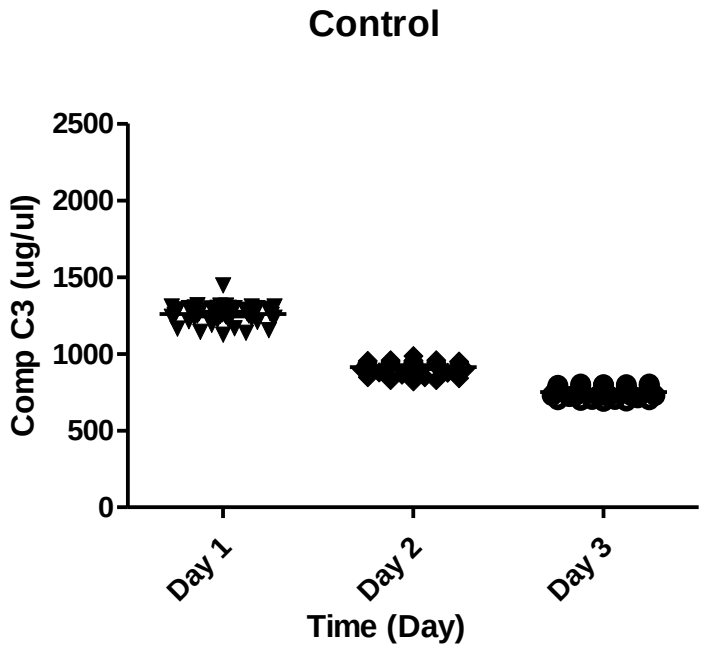
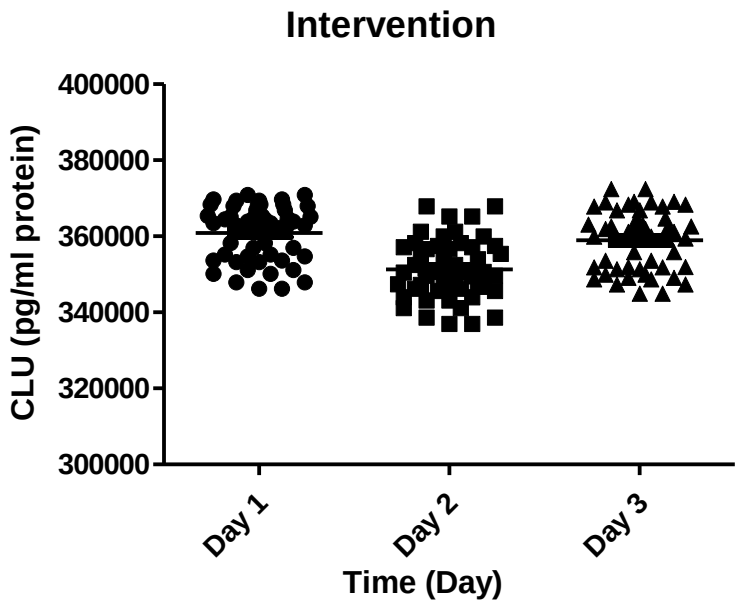


Figure 19: Mean of Comp C3 concentration in (a) Intervention and (b) Control group.

(a)



(b)

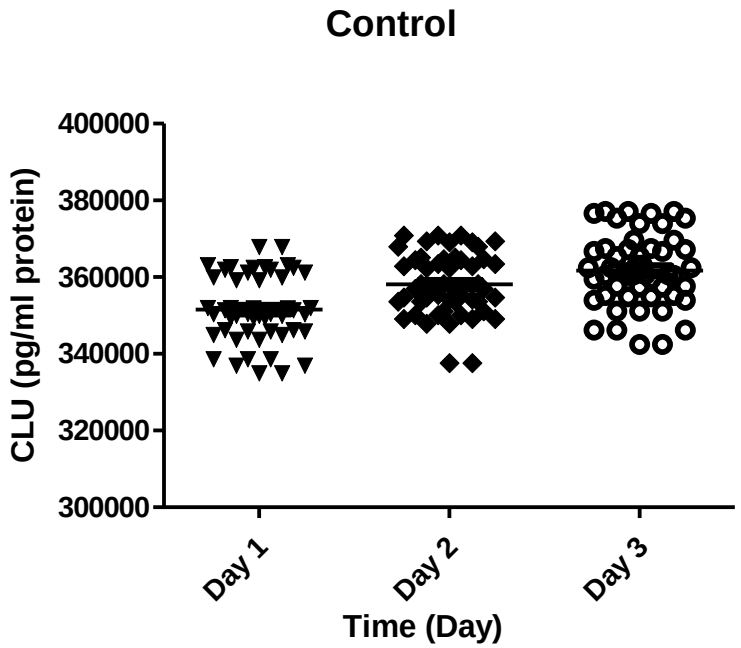
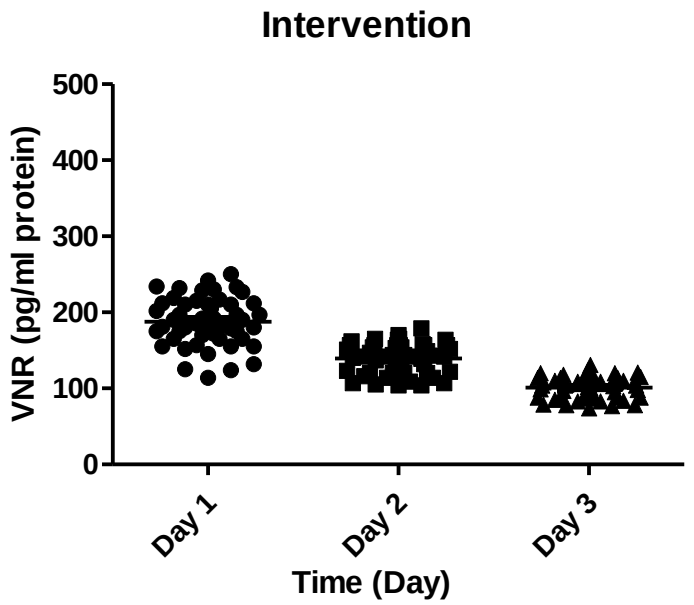


Figure 20: Mean of CLU concentration in (a) Intervention and (b) Control group.

(a)



(b)

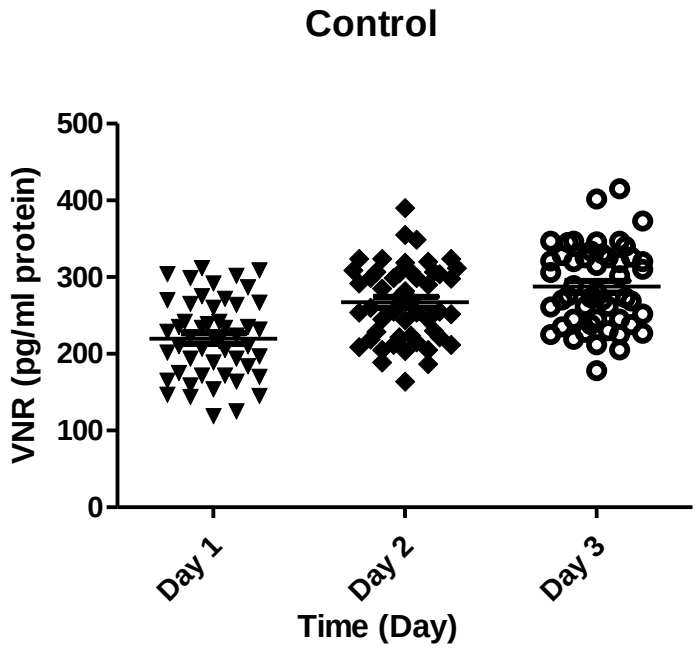
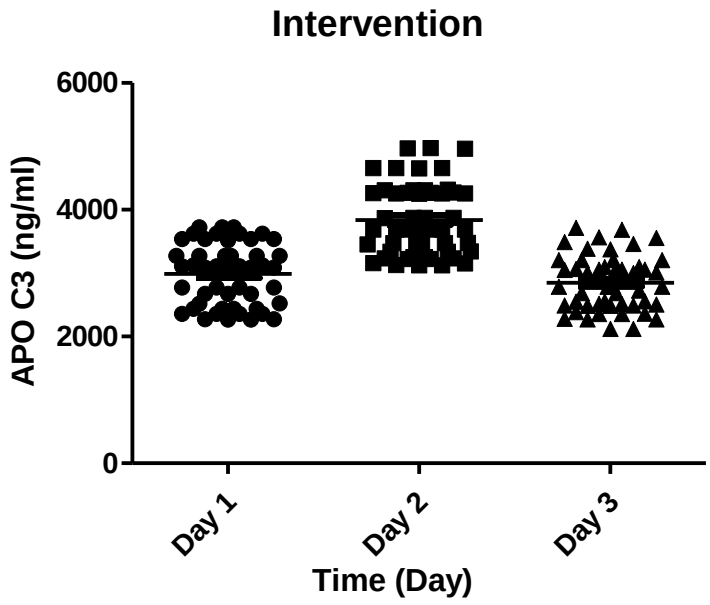


Figure 21: Mean of VNR concentration in (a) Intervention and (b) Control group.

(a)



(b)

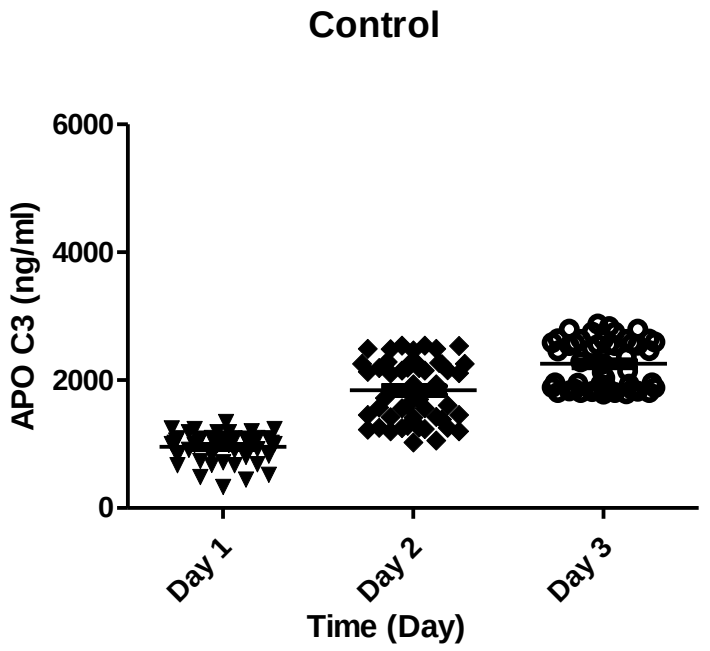


Figure 22: Mean of APO C3 concentration in (a) Intervention and (b) Control group.

Table 9: Relative expression of serum proteins analyzed by ELISA

Protein	Intervention (Fold changes relative to the previous day)		Control (Fold changes relative to the previous day)	
	Day 2	**Day 3**	**Day 2**	**Day 3**
VDBP	-1.05 (p< 0.0001)	+1.04 (p< 0.0001)	+1.05 (p< 0.0001)	+1.09 (p< 0.0001)
AHSG	-1.05 (p< 0.0001)	+1.04 (p< 0.0001)	-1.03 (p< 0.0001)	-1.05 (p< 0.0001)
Comp C3	+1.59 (p< 0.0001)	-1.31 (p< 0.0001)	-1.38 (p< 0.0001)	-1.22 (p< 0.0001)
CLU	-1.03 (p< 0.0001)	+1.02 (p< 0.0001)	+1.02 (p< 0.0001)	+1.01 (p< 0.0001)
VNR	-1.35 (p< 0.0001)	-1.38 (p< 0.0001)	+1.22 (p< 0.0001)	+1.08 (p< 0.0001)
APO C3	+1.28 (p< 0.0001)	-1.35 (p< 0.0001)	+1.93 (p< 0.0001)	+1.22 (p< 0.0001)

+ Increase in expression; - decrease in expression

CHAPTER 5

DISCUSSION

CHAPTER FIVE: DISCUSSION

The World Health Organization (WHO) estimated that more than 70 % of the worldwide population who are at risk of dengue lives in the Southeast Asia region and Western Pacific regions (WHO, 2009). As such countries like Malaysia, Thailand, Vietnam, Indonesia and the Philippines frequently have high dengue cases amongst its populations. In fact, during the first eight months of 2010, the number of dengue cases reported in countries such as Thailand, Philippines, Vietnam, Sri Lanka, and Indonesia ranged from 24,000 to 80,000 (Kwok, 2010). During the same period, Malaysia reported 46,171 cases with 134 deaths. Both Selangor and the Federal Territories reported the highest number of cases in Malaysia. The number of dengue cases in Selangor in 2010 was 16,367 cases with 45 fatalities. Among the districts in Selangor, the district of Petaling reported the highest incidence with 5,111 cases, followed by Hulu Langat (4,852 cases), Gombak (3,107 cases) and Klang (1,788 cases). Selangor reached its highest incidence of dengue in 2008 with 21,262 cases and 46 fatalities (Nusyirwan, 2010). While for the whole of Malaysia, a total of 49,335 dengue cases was reported in 2008, out of which, 46,517 were classified as DF cases and 2,818 cases were classified as DHF (Mudin, 2009). In 2009, 41,486 cases were reported, with 88 reported death, 38,749 cases were classified as DF and 2,737 cases were classified as DHF (MOH Malaysia, 2010).

The importance of dengue serotype surveillance for monitoring dengue outbreaks has been reported by Thayan *et al.*, 2001 and Hilaire & Greenidge, 2008. Among the reasons associated with an increase in dengue cases are the change in circulating dengue serotype and genotypes among the population. According to reports from the Ministry of

Health Malaysia, DENV-3 was the dominant serotype in the early 90s until it reached its peak in 1993 and it then reemerged in 2001. DENV-1 was dominant from 2003 until 2006 and in 2007; DENV-2 emerged as the dominant serotype in the country (Ministry of Health, 2010). In 2009, DENV-1 and DENV-4 were no longer isolated and only DENV-2 and DENV-3 was found to be co-circulating in the country (Mia *et al*., 2013). In the present study, we found a predominance of DENV-1 among dengue patients admitted at Hospital Tunku Ampuan Rahimah, Klang between the periods of 2010-2011 and this can be correlated as the situation in Selangor. We also found the presence of other co-circulating strains such as DENV-2, DENV-3 and DENV-4 in this hospital. A recent study by Ahmad Nizal *et al.* (2012) which was carried out in Negeri Sembilan also found a similar predominance of DENV-1 serotype among their patients.

In a study conducted in Bangkok, Thailand, different types of DENV were detected circulating between 2004 and 2010. DENV-1 was the predominant serotype in 2004 (56.41%) followed by DENV-4 in 2007 (50%), DENV-1 in 2008 (57.41%), and DENV-2 in 2010 (38.7%) (Pongsiri, Themboonlers, & Poovorawan, 2012). In addition, the researchers found that DENV-2 and DENV-3 usually cause the more severe form of dengue. They also reported that DENV-4 causes milder disease in primary DENV infection (Fried *et al*., 2010). Another study showed that DENV-2 and DENV-3 were associated with severe dengue disease, DHF, whereas DENV-4 is found primarily in secondary dengue infection (Nisalak *et al*., 2003). A study in India also found that DENV-2 and DENV-3 were causing more severe dengue infections whilst DENV-1 seems to be the mildest serotype (Gupta *et al.*, 2006).

During the outbreak of dengue in Singapore in 2005, a total of 14,006 cases were reported with 27 deaths. DENV-2 was determined to be the dominant serotype during that outbreak (Koh *et al*., 2008). DENV-1 then became the dominant serotype in 2006, and continued to remain as the dominant serotype during another outbreak in 2007 (Lee *et al*., 2010). However, in 2007, the predominant circulating serotype switched back from DENV-1 to DENV-2, and the number of dengue notifications in that year was almost three-fold higher than that of 2006. Between the period of 2007 and 2011, DENV-2 was the predominant circulating serotype in Singapore (Ministry of Health, Singapore, 2012). There were no cases of death reported.

In Indonesia, DENV-2 was found to be the dominant serotype in Surabaya, the second largest city in Indonesia between 2003 to 2005. However, DENV-1 became the predominant serotype between 2008 to 2009 (Yamanaka *et al*., 2011). In 2009, the number of outbreaks in the country reached more than 150,000, but dropped by 50 % to around 75, 000 in 2010, and then down to 50,000 in 2011 (Yoshikawa & Kusriastuti, 2013; Gunawan, 2012). Among the possible reasons for the dropped in dengue cases were the change in circulating dengue serotype and genotype in the populations. Usually a particular dengue serotypes pre dominates the other strains over a certain period of time, which can last up to 2 to 3 years. As the population is sensitized to the prevalent dengue serotype and the development of herd immunity in the population may limit the transmission of the virus, a different dengue serotype takes over the pre dominance. This usually causes an increase in dengue cases due to the presence of un-sensitized population (Thayan *et al*, 2001).

In a 3-years surveillance study done in Kampung Cham, Cambodia, DENV-3 was the most predominant serotype (n= 234, 55.3%), followed by DENV-1 (n =115, 27.2%) between 2006 and 2008 (Vong *et al.*, 2010). Dengue outbreaks from 1995 to 1998 in Philippines were dominated by DENV-2 and DENV-3 while DENV-2 was the most frequently isolated serotype from 1995 to 2002. Besides DENV-2, DENV-1 was also commonly isolated from 1999 to 2002 (Salda *et al*., 2005).

Many researchers have associated severe dengue outbreaks with DENV-2. For example, Fried *et al.* 2006 reported that DENV-2 has been marginally associated with more severe dengue as there was an increase in DHF grade 1 cases as compared to DENV-1 while Guzman *et al.* (2008) noted that the Asian genotype of DENV-2 and DENV-3 resulted in more severe cases of DHF. This study has shown that the most dominant serotype in the Western (HTAR) part of Selangor was DENV-1 (34%). This corresponds with MOH Malaysia report where dengue cases are usually low when DENV-1 is the pre-dominating serotype. This could be the reason for the lower number of dengue cases and fatalities in Selangor in 2011.

Rapid diagnosis and identification of dengue serotype can provide useful information for early and accurate diagnosis. It is also important to understand clinical problems that may arise during the different phases of the disease. In acute dengue infections, initially there is a viraemic phase in the first few days of exposure where the dengue virus is present in blood before being neutralized by dengue virus specific antibodies. In this study, we have utilized a commercially-available point-of-care rapid diagnostic test to determine the dengue status of each patient before serotyping was done.

This rapid test is able to detect the dengue NS1 antigen and dengue IgM antibodies simultaneously. The usual dengue detection methods that are carried out in the laboratories in hospitals are either dengue IgM antibodies (MAC-ELISA) or dengue NS1 antigen detection separately. Usually dengue IgM ELISA is most sensitive but may not be a suitable test at an early onset as the levels of antibodies may not be sufficient enough to be detected as IgM does not become detectable until 5 to 10 days after the onset of illness in primary dengue virus infection or until 4 to 5 days in the case of secondary infection (Innis, 1989; Gubler, 1998). In addition, specialized techniques such as dengue viral isolation and multiplex real-time RT-PCR to determine dengue serotypes are only available in research or surveillance laboratories (Wang & Sekaran, 2010). However, all of these methods have their advantages and disadvantages. For example, virus isolation is time consuming and requires at least 6–10 days for the virus to replicate (Shu & Huang, 2004). It also requires cell culture facilities or mosquito colonies, which need good maintenance of the laboratory (Harris *et al*., 1998). Molecular techniques such as multiplex real-time RT-PCR needs specialized equipment and reagents to be carried out and may not be feasible in resource-limited settings. Hence a test that is able to detect the virus and antibodies simultaneously will be most suited to be utilized in the laboratories.

The rapid test employed in this study detects both dengue virus NS1 antigen and differential IgM/IgG antibodies. NS1 is a highly conserved protein that is found from day 1 to day 9 after the onset of fever, either in primary or secondary infection (Alcon *et al*., 2002; Young, Hilditch, Bletchly, & Halloran, 2000). IgM are detectable in primary dengue infection on the 3rd to 5thday of illness, and may last up to 2-3 months (Wang & Sekaran, 2010). IgG usually appear on the 14th day of infection and lasts a lifetime. However, in

secondary infection, IgG appears on the first two days after infection, thus patients with secondary infection will usually have a positive IgG result (Shu & Huang, 2004). Our studies show that 49% of all dengue patients recruited tested positive for SD Duo NS1 Ag; and 83% patients tested positive for SD Duo IgM/IgG. This shows that all the patients recruited for the study were confirmed to have dengue infection by laboratory assays. About half of them were actually in the viraemic phase based on NSI Ag test while the rest had dengue IgM/IgG detected.

At present, the technology of real-time RT-PCR has been used by many researchers to detect all serotypes of DENV RNA in human samples (Chutinimitkul *et al*., 2005; Johnson *et al*., 2005; Leparc-Goffart *et al*., 2009; Yong *et al*., 2007). According to Poersch *et al*. (2005), real-time RT-PCR is more sensitive, detecting more than four times as many acute-phase samples as the nested RT-PCR. In this study, real-time RT-PCR was used to detect dengue virus serotypes. SYBR® Green was used as fluorescent indicator because it can easily detect the products amplification. This is because the dye intercalated in the minor groove of double-stranded DNA. The percentages of G+C content in each dengue genotype are different, which makes the Tm values differ from each other. The melting curve analysis results shows that all four types of dengue serotypes could be identified based on its distinctive Tm values. The melting curve analysis in this study showed that mean of Tm values for each dengue serotypes were almost similar in both the I and C groups. In group I, the Tm value for DENV-1 was 85.1 °C, DENV-2 was 82.5 °C, DENV-3 was 85.9 °C and DENV-4 was 83.1 °C. In group C, the mean of Tm value for DENV-1 was 85.0 °C, DENV-2 was 82.5 °C, DENV-3 was 85.8 °C and DENV-4 was 83.4 °C.

It has been established that real-time RT-PCR using SYBR® Green is more reliable, flexible, simpler and costs cheaper compared to other PCR methods (Papin *et al*., 2004). The SYBR® Green real-time RT-PCR that was carried out revealed the presence of individual melting curve. The specificity of the PCR product was analyzed by looking at its Tm. The Tm for each sample was used to identify the dengue serotype. Samples with the same Tm were considered as belonging to the same serotype (Yong *et al*., 2007). However the disadvantages in using SYBR® chemistry is the reduced specificity as point of determination of unknown samples is entirely dependent on the melt curve of standards used and any slight variation of melt curve of unknown samples from the standards used will result in an anomaly and must be verified by sequencing. The solution will be by using Taq man chemistry where four different probe chemistry for each of the four dengue serotypes are utilized in a single reaction. However, this will incur increased costs as the probes can be expensive and optimization is needed for multiplex real-time probe chemistry.

According to Yong *et al.* (2007), dengue serotypes can be distinguished by both serological and molecular methods. Previously, dengue detection was done by viral isolation in cell cultures followed by direct detection using IF, which could be quite tedious. The viral isolation is time consuming as it takes at least 7 days to complete. There is also a tendency of interference from the antibody during viral isolation when its level begins to rise on Day 3 after onset. This will result in low sensitivity of viral isolation. PCR-based techniques on the other hand are quick and sensitive. According to Yong *et al.*

(2007), the rate of detection in real-time SYBR® Green is higher compared to other PCR-based techniques, even with the presence of anti-dengue antibodies.

In serological diagnosis, the IgM Capture ELISA revealed that IgM antibodies were detected in 88 out of 100 dengue patients, indicating that 88% of the patients have recent infection. IgM antibody is the first immunoglobulin isotype to appear during primary infection, which response to dengue antigens followed later by IgG response. During a secondary infection, high levels of IgG will be detected even in the acute phase and it rise dramatically over the following two weeks, diminishing IgM response. According to Sadon *et al*. (2008), serology is often inaccurate due to cross-reactivity among flavivirus.

Proteomics technologies are used to identify new targets for therapeutics, development of novel biomarkers for diagnosis and early detection of disease. 2D-GE is the gel-based method used to separate the complex mixtures of protein. Though it may provide several types of information, including quantity, molecular weight, p*I* and possible post-translational modifications, it may not detect low abundant and hydrophobic proteins (Chandramouli & Qian, 2009). Thus, to overcome this problem, a new technology is utilized that enables enrichment of low abundance proteins in a sample such as serum that has a wide dynamic range spanning more than ten orders of magnitude in concentration (Anderson and Anderson, 2002).

In this study, the high-abundance proteins in serum such as albumin and IgG were decreased without immunodepletion by using the ProteoMiner™ kit, followed by the analysis of gels using the 2D-GE technique. The high-abundance proteins saturate their high affinity ligands and excess proteins were washed out during the procedures. On the other hand, the medium- and low- abundance proteins are concentrated on their specific affinity ligands, thereby decreasing the dynamic range of proteins in the sample.

Our study demonstrated that 2D-GE technique could be used to differentiate the altered expression of proteins in sera of dengue patients that were treated with CPL juice and those who were not treated. The 2D-GE images revealed the presence of several protein spots that were differentially expressed among group I and group C. A total of six proteins were identified, where four of them were down-regulated and two of them were up-regulated after the treatment of CPL juice. The down-regulated proteins were VDBP, AHSG, CLU and VNR. The up-regulated proteins were Comp C3 and APO C3. All of these proteins have been reported to have some association in the pathogenesis of dengue.

VDBP was previously known as the group-specific component of serum (Gc-globulin) (White & Cooke, 2000). It is synthesized in hepatic parenchymal cells in a constitutive manner. It is found in plasma, ascetic fluid, cerebrospinal fluid, seminal fluid, saliva, breast milk and urine. This protein belongs to the albumin superfamily of binding proteins such as albumin, α-albumin and α-fetoprotein (Gomme & Bertolini, 2004). Human serum VDBP is a monomeric glycoprotein, where its exact size depends on its

glycosylation state (White & Cooke, 2000). VDBP binds vitamin D and transports them throughout the whole body (Gomme & Bertolini, 2004) and also protects vitamin D metabolites from hydroxylase-mediated catabolism (Møller *et al*., 2013). It also binds, solubilize and transport two types of sterols; 25-hydroxyvitamin D (25(OH) D; calcidiol) and 1, 25-dihydroxyvitamin D (1, 25(OH) 2D; calcitriol). Both of these sterols are the major circulating metabolite and the most active metabolite of the vitamin, respectively (White & Cooke, 2000). Bouillon *et al.* (1981) discovered that the concentration of VDBP was increased during pregnancy. Møller *et al*., (2013) also discovered that estrogen component of hormonal contraceptives may increase VDBP synthesis or decrease its catabolism. Another study has proved that VDBP and vitamin D receptor gene polymorphisms were strongly associated with asthma characteristics as well as vitamin D level in Egyptian children (Ismail, Elnady & Fouda, 2013).

According to Gomme and Bertolini (2004), VDBP binds g-actin and prevents further nucleation and polymerization in actin scavenger system, play roles in chemotaxis and acts as a switch in turning macrophage activity on at sites of infection and inflammation and instigating cell death when their presence is no longer required. Loke *et al.* (2002) had suggested that VDBP may be associated with the pathogenesis of DF, by activating monocytes, stimulating cellular immune response and suppressing immunoglobulin production and lymphocytes proliferation. The plasma concentration of VDBP is stable from birth and will increase up to 50% in high oestrogen states. However, the levels are significantly reduced in severe hepatic disease (Haddad, 1995). According to Albuquerque *et al*. (2009), the level of this protein is significantly increased in DF patients as compared to healthy patients. Our study also showed that the level of this protein was

increased up to two-fold in the control group. Interestingly, the level of this protein was found down-regulated up to two-fold after the patients were given the CPL juice, which indicates that the juice might able to decrease the level of VDBP back to its normal concentration, thus assisting the recovery of DF patients.

AHSG was also down-regulated in dengue patients as shown in this study. This highly abundant protein is synthesized in the liver and osteoblasts. AHSG belongs to the cystatin super family. It is one of the negative acute phase proteins (APPs), where the levels of this protein decrease when an inflammation or infection occurs (Lebreton *et al*., 1979). It has a molecular weight of 51,000 and circulates in the plasma as two polypeptide chains; A with 282 amino acids and B with 27 amino acids. These bonds are covalently held together through disulphide bond. According to the study performed by Lebreton *et al*., (1979), AHSG serum level was greatly decreased in patients who developed an acute inflammatory process after a severe bacterial infection. It was assumed that the decreased levels were caused by impaired hepatic synthesis. The concentration of AHSG was also found to be decreased in human serum due to malnutrition and Paget's disease of bone (Yang *et al.,* 1991). Saroha and colleague (2012) found that the level of plasma AHSG was decreased by two-fold in rheumatoid arthritis patients as compared to normal controls. It is believed that AHSG protein plays an important role in the development of bone marrow, brains, gonads and liver (Arnaud & Kalabay, 2002). Yang *et al.* (1991) suggested that AHSG enhanced endochondral bone formation by elevating alkaline phophatase activity in the cartilage. AHSG could prevent excessive bone mineralization and can also act as protease inhibitor. A study that was done in Germany had suggested that AHSG inhibits the *de novo* formation and precipitation of the apatite precursor mineral, basic calcium phosphate (BCP), only

transiently (for several hours), and does not dissolve BCP once it is formed. Therefore, AHSG can inhibit undesirable calcification in circulation without inhibiting bone mineralization. The inhibitory activity of serum proteins on apatite formation was largely reduced after the specific depletion of AHSG from the serum (Schäfer *et al*., 2003).

AHSG was found to be a potential biomarker for clinical serologic screening of breast cancer (Yi *et al.,* 2009). Kuakarn *et al*. (2013) believed that AHSG and complement C3c are potential serum pre-treatment biomarkers in predicting sustained virological responses in chronic hepatitis B patients treated with peginterferon alpha-2b. Lewis (1983) has reported that AHSG has a role as a modulator of the immune responses.

According to Silva *et al*. (2013), during dengue infection, most of the identified NS1-interacting partners are the APPs, which includes AHSG, actively involved in the acute phase response (APR). The APR plays an important role in limiting hepatic tissue injury. This is because the level of IL-6 will increase during the infection, thus increasing the concentration of positive APPs and decreasing the concentration of negative APPs. The decrease level of AHSG during infection is controlled by several cytokines, which act directly on the expression of the gene through its promoter region. So, when a DF patient starts to recover, the level of positive APPs was presumed to decrease and the level of negative APPs was presumed to increase. In this study, initially, the level of AHSG detected was decreased 24 hours after the treatment. However, the AHSG level started to increase on Day 3. This may indicate that the juice was able to increase the level of AHSG in DF patients in order for the patient to recover faster, but it may take a while for the protein to react towards the treatment. In the control group, this level of this protein

decreased on the second and third day, which may suggest that recovery phase has not commenced yet.

This study also found that administration of CPL juice resulted in two-fold increase of Comp C3 levels in contrast to the control group which recorded two-fold decrease of expression. Studies have indicated that the complement system provides protective immunity among dengue patients, especially in the context of ADE (Mehlhop, 2007). C1q was observed to limit ADE *in vivo.* On the other hand, *in vitro* studies using DENV NS1 protein shows that the viral protein binds to C4 and C1s to antagonize complement activation (Avirutnan *et al*., 2010). The role of the mannose-binding lectin pathway of the complement system in modulating DENV infections have been suggested (Sujan, 2012). There is also evidence that levels of Comp C3 correlated directly with platelet count but inversely followed C3a levels among severe dengue patients (Nascimento, 2009). The utilization of Comp C3, C3a and C5a production was related to thrombocytopenia, seen among severe dengue patients (Lin, 2001; Srichaikul & Nimmannitya, 2000; Nascimento, 2009).

In dengue infection, it was reported that during the time of defervescence, high levels of C3a and C5a were first found in plasma followed by a steep reduction among patients with severe dengue infection/fever (Martina, 2009), suggesting marked consumption of the complements to aid the opsonization of the antigen-antibody complex. Comp C3 protects against viral infection by priming adaptive B and T cell responses and triggering release of C3a and C5a which are anaphylatoxins and which can opsonize

viruses for phagocytosis and destruction of macrophages (Avirutnan *et al.,* 2010). In addition, C3a has also been shown to activate platelets and aid its adhesion (Martina, 2009). *In vitro* studies have also demonstrated the role of complement proteins in regulating dengue virus infections where C4 and C5 antagonize complement. An increase in activity of Comp C3 protein suggest efforts to aid the productions of C3a to firstly remove the pathogen by opsonization and secondly to activate platelets and their adhesion qualities.

Usually there is depletion of platelets in the initial stage of dengue infection as a result of being consumed during formation of dengue antigen-antibody complex and maybe due to increased platelet adherence to dengue virus-infected endothelial cells (Krishnamurthi *et al*., 2001). Dengue antibodies usually target the PF4-heparin complex of the platelet surface, resulting in close contact with the Fc_{δ}RIIa, enabling increased binding and thus their depletion. We hypothesized that the two-fold increased in Comp C3 production as a result of CPL juice consumption had caused an increase in the levels of C3a molecules. These molecules had assisted in removing dengue viral particles and hence affect antigen-antibody-platelet complex. As a result, less platelet is taken up for the antigen-antibody complex. Subenthiran *et al.* (2013) found that there was a marked increase in platelet count among dengue patients given CPL juice.

CLU, also known as Apolipoprotein J (ApoJ), is a secreted heterodimeric glycoprotein of 75-80 kDa (Jones & Jomary, 2002). It encodes an mRNA of approximately 2 kb which directs the synthesis of a 449-amino acid primary polypeptide chain. CLU is a major protein in physiological fluids including plasma, milk, urine, cerebrospinal fluid and

semen. The concentration of this protein was found with the highest level in the testis, liver, stomach, brain and epididymis (Jordan-Starc *et al.*, 1992). CLU is expressed in many organs at fluid-tissue interface which indicate a role in protecting cell membranes from the injurious effects of the secretion from gastric juice, bile, urine and pancreatic juice (Jordan-Starck *et al.*, 1992). The role of CLU includes sperm maturation, complement defense and skeletal components of sperm tail in rat. CLU also initiates luteolysis, facilitate sperm-ovum interaction and involve in the transportation of lipid between cells. CLU is also involved in the packaging and stabilization of peptide hormones or in the facilitation of exocytosis (Rosenberg & Silkensen, 1995). CLU is also a part of the fluid phase inhibitor of the terminal pathway of the complement system (Murphy *et al.*, 1989).

Kurosu *et al*. (2007) showed that the interaction between NS1 proteins and the human CLU may be involved during plasma leakage caused by dengue virus infection via the regulation of complement activity. The NS1 interferes with the inhibitory function of CLU at the terminal pathway. As a result, the complement system is enhanced and triggers plasma leakage. They had proposed two possibilities on how the interaction of NS1/CLU affects the terminal pathway. Firstly is that these complex may be removed after the clearance of viral proteins by antibodies against NS1, which result in the decrease of CLU levels. Secondly, they proposed that NS1 may inhibit the interaction of CLU with C7, which is associated with CLU under conditions where the complement system is inactive. The CLU-C7 interaction inhibits the subsequent formation of the complement system. The removal of this inhibition triggers the formation of the complement system. Albuquerque *et al*. (2009) reported that the levels of CLU increased in DF patients compared to healthy individuals. They described that CLU inhibited the hemolytic activity of the MAC of the

complement system and the increasing levels of this protein has been related to cell death. In our study, this protein was shown to be up-regulated in the control group while the level of CLU in patients that has been treated with CPL juice was down-regulated. Hence down regulation of CLU among dengue patients suggest recovery phase as a result of increased complement activity upon consumption of CPL juice.

Another down-regulated protein found in dengue patients in this study is the VNR. VNR is a multifunctional serum protein, produced in the liver (Felding-Habermann & Cheresh, 1993). Schvartz, Seger & Shaltiel (1999) reported that VNR, also known as the S-protein, encodes for 459 amino acids which are preceded by a 19-amino acid signal peptide. Its molecular weight is 75 kDa and its gene is composed of 4.5-5 kb pairs. It contains eight exons and seven introns. The major part of VNR molecule accommodates six hemopexin repeats. The concentration of VNR in plasma is 200-400 µg/ml and constitutes 0.2 – 0.5 % of total plasma protein (Preissner & Seiffert, 1998).

VNR promotes cell adhesion and spreading (Jenne & Stanley, 1985). This protein has shown to have consensus sequences for phosphorylation by various protein kinases. VNR multimers were shown to exert preferential binding to several ligands. Besides involving in the formation and the dissolution of blood clots, VNR has also shown to act as an inhibitor of the cytolytic reactions of the terminal complexes of complement and of perforin. Bhakdi *et al*. (1988) that this protein is associated with cytolytic membrane-bound C5b-9 complexes. VNR binds the C5b-7 complex and stops it from being inserted into the cell membrane (Milis, Morris, Sheehan, Charlesworth, & Pussell, 1993). A cell surface protein, CD59 (protectin), inhibits MAC formation on the cell surface by blocking the

binding of C8 and C9 to the C5b-7 complex. When a dengue infection occurs, complement system will be activated in order to opsonize the viruses. The existence of VNR will interrupt this system, thus will cause the infection to be more severe. In our study, patients that were treated with CPL juice show a decrease in the level of VNR while the patients that were not treated showed an increase level in VNR. Hence, down-regulation of VNR among patients who were given CPL juice suggests efforts to minimize the suppression function of the protein. The lesser interruption from this protein, the more efficient the complement system will be. This gives higher chances to the complement system to complete its system, killing more viruses that exist in the patients' body system thus leading to the patients' recovery.

Besides Comp C3, APO C3 was also found to be up-regulated in the Intervention group. APO C3 is an 8.8 kDa glycoprotein (Chan *et al*., 2008) that is secreted by the liver and intestine. It is controlled by positive and negative regulatory elements that are spread throughout the gene cluster (Jong, Hofker, & Havekes, 1999). This protein gene is localized at human chromosome 11 with Apo A-I (Bruns, Karathanasis, & Breslow, 1984). There are at least 3 forms of APO C3; APO $C3_0$, APO $C3_1$, and APO $C3_2$. These forms are categorized according to their moles of sialic acid present (Malmendier *et al*., 1988) and their entire sugar moiety (Ito *et al.,* 1989). APO C3 is a major component of triglyceride-rich lipoproteins and high-density lipoprotein.

Earlier studies have showed that APO C3 inhibits the hydrolysis of triglyceride by both lipoprotein lipase (LPL) (Brown & Baginsky, 1972) and hepatic triglyceride lipase. Ginsberg and colleagues (1986) also showed that *in vitro* and *in situ* studies suggested

another role for APO C3; *i.e* as an inhibitor of Apo E-mediated hepatic uptake of chylomicron remnants the plasma and very low density lipase. Thus, APO C3 may play a regulatory role in the catabolism of triglyceride rich lipoproteins. Wang *et al.* (1985) had measured and correlated the LPL inhibitory activity with the concentration of Apolipoproteins A-I, A-II, B, C-II, C-III, D, and E. They discovered that the plasma APO C3 levels were correlated positively with the LPL inhibitory activity, which suggested that APO C3 may represent a physiologic modulator of LPL activity levels. Smith *et al*. (2009) had found significant associations between APO C3 genotype and cognition in people with diabetes, which may be mediated through established vascular disease risk factors. Duivenvoorden *et al*. (2005) showed that APO C3 in mice are more sensitive to diet-induced obesity followed by a more aggravated development of insulin resistance compared with their control littermates. Therefore, they concluded that APO C3 may be a potential therapeutic target for the treatment of obesity and insulin resistance. Another study showed that APO C3 were associated with nonalcoholic fatty liver disease and insulin resistance in a cohort of Asian Indian men and non-Asian Indian men (Petersen *et al.*, 2010). A study has also shown that APO C3 experiences regulation at the hepatic mRNA as well as the plasma protein levels (Navarro *et al*., 2005). However, the correlation of this protein in dengue immune-pathology has not been established.

ELISA was performed for all six proteins in order to validate the results obtained from the 2D-GE part. Generally, all the proteins were regulated in similar pattern where an average 2D-GE profile shows 2-fold regulations whereas ELISA data reveals about 1.5-fold regulation.

CHAPTER 6

CONCLUSION

CHAPTER SIX: CONCLUSION

Till date, in terms of medical procedure for hospitalised patients, there is no effective vaccine or anti-virals available for the treatment of dengue infections. Patients are managed according to symptoms presented and thus it can be challenging for patients with severe dengue infection who present with haemorrhagic manifestations.

Unconventional methods including the use of natural products are being evaluated for properties of anti-dengue or as immune regulators. In Malaysia, the locals have been using CPL juice as a method to treat dengue. However, no scientific evidence is currently available on the mechanism of CPL juice for dengue infection.

Therefore, in this study, we investigated the use of CPL juice as a treatment procedure for dengue infection by using 2D-GE and MS analysis to identify the expressed proteins that were involved in the recovering process. We correlated these differentially expressed proteins with dengue pathology and immune modulation. We also determined the dengue serotypes among patients recruited in this study in order to monitor changes in dengue serotype in affected regions as any change in serotype distribution may result in an outbreak.

This study has shown that all four serotypes were present with DENV-1 was the most predominant dengue serotype. Secondly, six proteins were identified to be differentially expressed; two were up-regulated and four proteins were down-regulated after the treatment of CPL juice. The up-regulated proteins were Comp C3 and APO C3 while

the down-regulated proteins were VDBP, AHSG, CLU and VNR. Comp C3 protein is essential for platelet regulation and have been shown to be up-regulated upon consumption of CPL juice. In addition, two other proteins; VNR and CLU have been shown to be down-regulated. The functions of these proteins were also linked to complement regulations as this affect platelet production.

In conclusion, CPL juice has the potential to be used as a supplement for the treatment of dengue patients. This study suggests that most of the proteins that were found have some correlation with dengue pathology and immune modulation. We propose the use of CPL juice as a complementary therapy in addition to the current dengue patients' management.

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APPENDICES