DISSERTATION

METABOLOMICS-BASED DIAGNOSIS AND PROGNOSIS OF DENGUE VIRUS INFECTIONS AND NS1 ANTIGEN DETECTION FOR DIAGNOSIS AND SURVEILLANCE IN HUMANS AND MOSQUITOES

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2013

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ABSTRACT

METABOLOMICS-BASED DIAGNOSIS AND PROGNOSIS OF DENGUE VIRUS INFECTIONS AND NS1 ANTIGEN DETECTION FOR DIAGNOSIS AND SURVEILLANCE IN HUMANS AND MOSQUITOES

Dengue (DEN) is a mosquito-borne viral disease of significant public health importance. There are currently no commercialized vaccines or accepted pharmacological treatments for DEN disease, making mosquito surveillance critical for the prevention of outbreaks of this disease. *Aedes aegypti* is the principal vector of DENV, although *Aedes albopictus* and other *Aedes* species have been reported to be able to transmit it. Improved surveillance methods for DENV in mosquito populations would be of great value for public health and vector control programs and would provide better risk assessment of potential DENV infections in humans. Improved mosquito-based surveillance would also improve vector control programs by targeting areas at higher risk for ongoing or potential epidemics for vector control.

Non-structural protein 1 (NS1) detection by ELISA is a commercially available test with the ability to detect DENV NS1 protein in DENV infected samples. Studies were conducted to determine the ability of the NS1 antigen test to detect DENV in *Aedes aegypti* mosquitoes. The NS1 antigen detection test proved to be highly sensitive and specific for DENV-antigen detection in pools of mixed infected and non-infected mosquitoes under various field-simulated conditions and in different sizes of mosquito pools. This test could facilitate mosquito-based surveillance for early warning of DEN outbreaks.

The capacity of this test to diagnose human DENV infections in non-invasive clinical specimens, i.e., urine and saliva, was also investigated. NS1 protein detection in acute phase, non-invasive clinical specimens was found to be less sensitive than NS1 detection in serum samples.

Most dengue virus (DENV) infections are subclinical. The clinical manifestations of apparent infections range from DEN fever (DF), typically a self-limiting illness with fever and rash, to severe DEN hemorrhagic fever and shock syndrome (DHF/DSS). Upon presentation, there is no way to predict

whether or not the patient will experience DF or will progress to the severe form of disease (DHF/DSS). A sensitive and specific test that utilizes acute phase clinical specimens and that provides diagnosis of DENV infection as well prognosis of progression to severe disease is sorely needed. Such a test would permit identifying those patients destined for severe disease for appropriate patient management and therapeutic intervention.

An innovative metabolomics platform was used in these studies to determine a biosignature of small molecular biomarkers (SMBs) that can potentially differentiate patients with the most severe forms of disease from DF and non-DEN patients. SMBs that could potentially be diagnostic of DENV infections and prognostic of disease outcomes were identified in acute phase serum, saliva, and urine specimens obtained from DEN and non-DEN patients from Mexico and Nicaragua. Using acute phase serum specimens, a panel of six candidate SMB compounds was identified by tandem liquid chromatography-mass spectrometry (LC-MS/MS); five of these six biomarkers differentiated DF patients from those with DHF/DSS and have potential to diagnose and predict DEN disease or DEN severity (DHF/DSS) outcomes. Two candidate SMB compounds were identified in urine and one in saliva that could potentially be used for diagnosis and prognosis of DEN infections.

Tandem mass spectra of candidate compounds and commercial standards were obtained and compared to identify the SMB metabolites. Some of the SMB identities were confirmed by using the NIST (National Institute of Standards and Technology) or METLIN spectrum libraries.

The biochemical nature of the identified metabolites included phospholipids, fatty acids, amino acids, nucleosides, and vitamin D. These SMBs have potentially important roles in DEN pathogenesis and in endothelial cell metabolism (these cells are among the principal target cells affected during severe DEN disease).

The immediate goal of this dissertation research was to identify a biosignature panel of LC-MS/MS-identified candidate SMB metabolites that differentiate the DEN disease diagnosis groups

(DHF/DSS, DF, and ND) and that have potential for diagnosis and prognosis of DENV infections using acute phase serum and non-invasive clinical specimens. However, it is unlikely that LC-MS/MS technology will be applicable in the front-line clinics where DEN patients first present. Thus a long-term goal of the research project is to select a subset of these pathogenically and physiologically relevant SMBs and then determine the potential for the metabolite analyte or a surrogate (e.g., a protein involved in the metabolic pathway conditioning the metabolic change) to be incorporated into diagnostic formats amenable to point of care tests (POC), such as ELISA based formats. A diagnostic algorithm incorporating results from such a POC test and conventional laboratory and clinical biomarkers could provide dramatically improved capability for diagnosis and prognosis of DENV infections and would be of immense value to physicians in managing patients.

ACKNOWLEDGMENTS

I am very thankful to my graduate committee members Drs. Beaty, Blair, Eisen, Loroño-Pino, Belisle and Prenni. Thank you so much for all your help, knowledge and support during the time I had the honor to meet you and work with you.

Thank you to the professors who have encouraged me and inspired me, especially to Charlie Calisher, Erica Suchman, Sandra Quackenbush, Joel Rovnak, Ann Hess, Chet Moore, Ken Olson and William Black.

Also, thanks so much to fellow and former graduate students, CSU, IDA and AIDL personnel, especially Irma Sanchez-Vargas, Claudia Molins, Meaghan Beaty, Susan Rogers, Tach Costello, Amber Rico, Aaron Phillips, Bethany Bolling, Tyler Eike, Corey Broeckling, Jackie Scott, Farah Vera, Krystle Reagan, Tony Schountz, Rushika Perera, Brian Cranmer, Ashli Simone, Cynthia Paquette, and Sebabatra Mahapatra.

I want to especially thank my advisors Drs. Beaty and Blair for the enormous amount of time and dedication you have spent in training me in virology, bio-politics, research, immunology, writing and for all the positive influence, and support you have gave me every single day of this past 5 years. All that will come handier than pockets in the underwear some day and I will be forever thankful to you for that.

And last but not least, thanks to my parents Clara and Donald, and to my wonderful husband Edward for love, courage, teachings, and support during this journey.

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CHAPTER 1

LITERATURE REVIEW

I.A. INTRODUCTION.

Dengue (DEN) is the most important arboviral disease. Dengue virus (DENV) is transmitted to humans by mosquitoes, and *Aedes aegypti* is the most important urban vector. Epidemic DEN fever (DF) and DEN hemorrhagic fever and shock syndrome (DHF/DSS) are major causes of morbidity and mortality throughout the tropical world and have overwhelmed public health and clinical care capacity in disease endemic countries.[1-3] The emergence of DF and DHF/DSS in the Americas has been a public health disaster. Approximately 30% of patients in Latin America are now progressing to severe DEN disease or DHF/DSS. [4, 5] The needs and opportunities to combat the rising tide of DEN are many. In the absence of vaccines and therapeutics, improved DENV surveillance and vector control are critical to prevent DENV infections in humans. Improved mosquito-based surveillance for DENV could provide early warning of impending epidemics and targeted interventions in areas with infected mosquitoes to prevent the outbreaks. Similarly, development of novel approaches to permit early identification of patients destined to progress to DHF/DSS would be of great value for public health practitioners and for clinicians managing patients. The ability to utilize non-invasive clinical specimens (e.g. – urine and saliva) for diagnosis and prognosis of DEN would also be of enormous value. Combining the two would potentially provide a true paradigm shift in DEN diagnosis.

The detection of non-structural 1 (NS1) antigen in acute phase serum specimens is commonly used to diagnose DENV infections.[6] The adaptation of this technique for detection of NS1 protein in non-invasive clinical specimens would be of great clinical value because of ease of collection of specimens, especially from young children. Adaptation of this technique for NS1 antigen-based detection of DENV in mosquitoes would increase the armamentarium for DENV surveillance in endemic areas, and it would be of great value for public health practitioners.

Metabolomics provides important SMB information regarding the disturbances of the cellular metabolism during DENV infection. This information could be exploited for understanding of DEN pathogenesis and developing diagnostics. There are measurable metabolic differences in DENV-infected cells and DENV infected humans.[7-9] The metabolic biosignature of measurable differences in infected and non-infected cells could provide opportunities for developing diagnostic and prognostic tests for disease outcomes and for understanding the pathogenesis of DENV infections. A panel of diagnostic small molecular biomarkers (SMBs) that differentiate dengue hemorrhagic fever/dengue shock syndrome, dengue fever and non- dengue (DHF/DSS, DF, and ND) cases and are predictive of the different disease states could be exploited to diagnose and predict the outcome of DENV infections and could help alleviate the public health burden of epidemic DEN. A metabolomics approach could also provide insight into the fundamental processes that condition the pathogenesis in DHF/DSS cases and into the underlying specific cellular metabolic pathways and pathogenic mechanisms that condition severe disease.

In this Literature Review, the medical importance, taxonomy, and molecular biology and replication of DENV, a member of the genus *Flavivirus* in the family *Flaviviridae*, will be reviewed, as will the origin, evolution, and phylogenetic relationships of DENV. DEN pathogenesis, tropisms, and diagnosis in serum, urine and saliva specimens are mentioned. Also included are brief reviews of the innate and acquired immune responses and the clinical outcomes of DEN disease. The second half of this chapter consists of a brief review of metabolomics, which includes an overview of untargeted and targeted analysis approaches, the instrumentation for data collection, and data analysis. The chapter concludes with an overview of lipidomics and eicosanomics and the importance of this discipline for the development of a panel of biomarkers for diagnosis and prognosis of infectious diseases.

I.B. FLAVIVIRIDAE AND THE GENUS FLAVIVIRUS; TAXONOMIC RELATIONSHIPS AND MEDICAL SIGNIFICANCE.

I.B.1 Flaviviridae. DENV belongs in the genus *Flavivirus*, family *Flaviviridae.* The flaviviruses have an unsegmented, single-stranded, positive sense RNA genome. The 5' end has a methylated nucleotide cap but there is no poly (A) tract at the 3' end of the genome. Some members of the family *Flaviviridae* have uncapped RNA and contain a 5' internal ribosome entry site to promote translation.

The *Flaviviridae* family (Figure 1) contains four genera; *Flavivirus* (e.g., yellow fever virus (YFV), West Nile virus (WNV), and DENV*, Pestivirus* (e.g. bovine viral diarrhea virus, classical swine fever virus)*, Pegivirus* (e.g. GB virus A, GB virus C, GB virus D) and *Hepacivirus* (e.g. hepatitis C virus, GB virus B). This family gets its name from the Latin word "flavus" (yellow), which refers to jaundice caused by YFV infection of the liver. YFV was the first virus of this family discovered. Viruses in the *Flaviviridae* family are small, enveloped RNA viruses with an average diameter of 45 nm, and those in the genus *Flavivirus* are typically transmitted primarily by arthropod vectors. [10, 11]

Viruses belonging to the family *Flaviviridae* are important in both human and veterinary medicine. The *Flavivirus* genus itself contains many viruses of medical and veterinary importance. Some viruses in this genus are neurotropic and major causes of encephalitis in humans and other animals; other members of the genus cause hemorrhage or acute liver damage in humans and animals. Viruses in other genera in the family *Flaviviridae* are major causes of hepatitis (e.g., the *Hepaciviruses*) and decreased productivity and animal losses (e.g., the *Pestiviruses*).

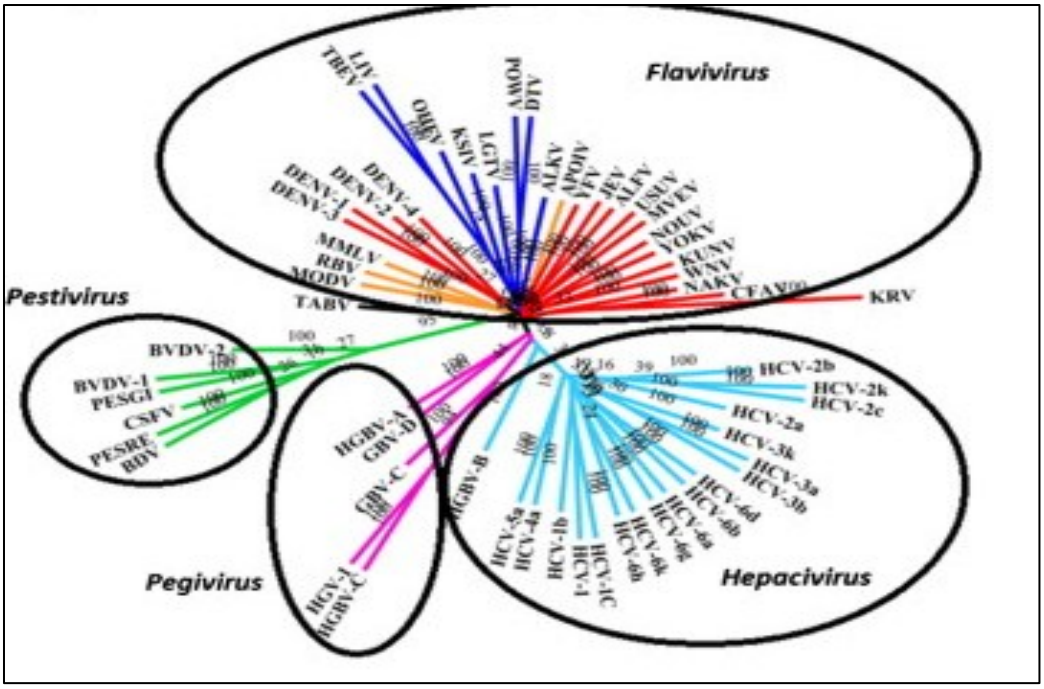


Figure 1. Family Flaviviridae phylogenetic tree. Flaviviruses transmitted by mosquitoes include DENV-1-4, yellow fever virus (YFV), Japanese encephalitis virus (JEV), Alfuy virus (ALFV), Usutu virus (USUV), Murray Valley encephalitis virus (MVEV), Kunjin virus (KUNV), and West Nile virus (WNV). Mosquito only viruses (not transmitted to vertebrates) include Nounane virus (NOUV), Nakiwogo virus (NAKV), cell fusing agent virus (CFAV) and Kamiti River virus (KRV). Flaviviruses transmitted by ticks include tick-borne encephalitis virus (TBEV), louping-ill virus (LIV), Omsk hemorrhagic fever virus (OHFV), Karshi virus (KSIV), Langat virus (LGTV), Powassan virus (POWV), deer tick virus (DTV), and Alkhurma virus (ALKV). The flaviviruses with no known vectors are Yokose virus (YOKV), Modoc virus (MODV), Rio Bravo virus (RBV), Montana myotis leukoencephalitis (MMLV), and Apoi virus (APOIV). The Pestiviruses include bovine viral diarrhea virus (BVDV-1, BVDV-2), pestivirus giraffe (PESGI), classical swine fever virus (CSFV), and pestivirus reindeer (PESRE). The Hepaciviruses included are hepatitis C virus (HCVs) and HGBV-B. The Pegiviruses include hepatitis GB virus (HGBV-A, HGBV-C), HGV-1, GBV-C and GBV-D. Taken from Kolekar, et al 2012. [12]

I.B.2. Flavivirus. The genus *Flavivirus* of the family *Flaviviridae* is composed of over 53 virus species officially recognized by the International Committee on Taxonomy of Viruses. *Flaviviruses* infect an interesting diversity of hosts, including birds, marsupials, primates and insects; there is significant clustering of viruses based on the affected reservoir host, especially in the cases of primates and birds (Figure 2).[13]

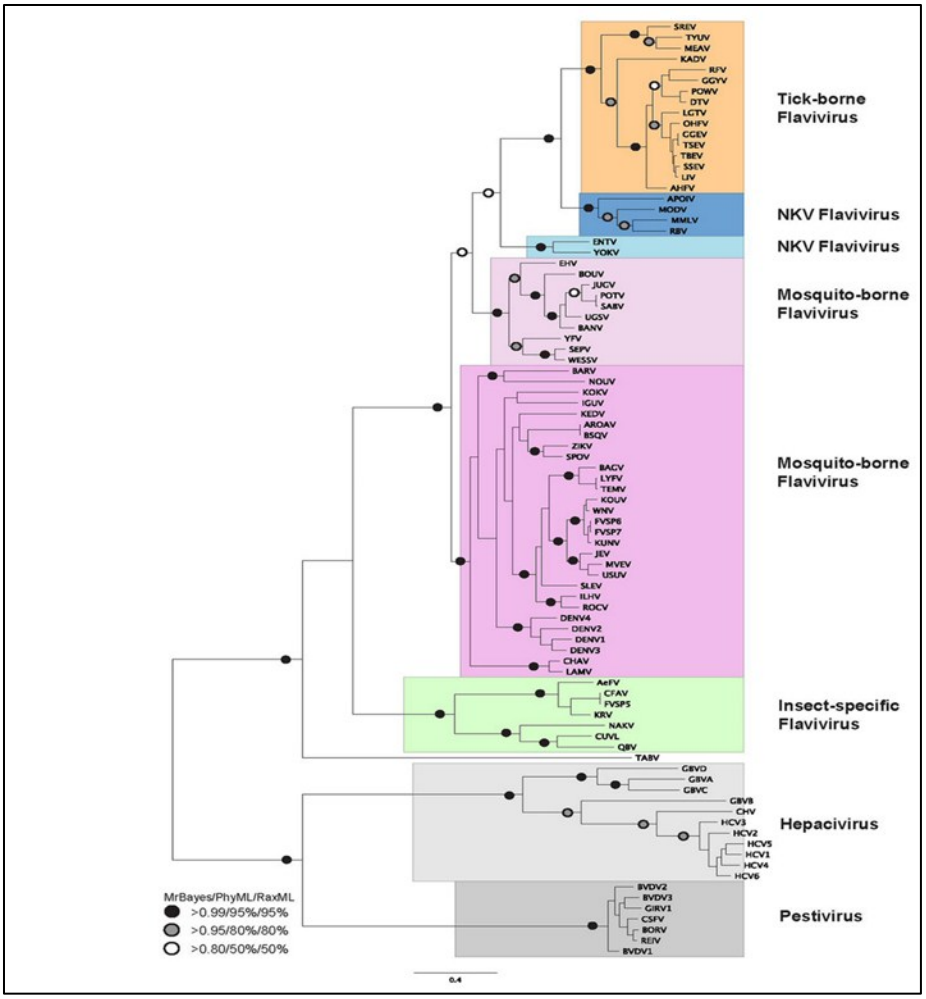


Figure 2. Genus Flavivirus phylogenetic tree. A rooted-phylogenetic tree. The viruses segregate into different phylogenetic branches. The numerical values at the bottom indicate statistical support for posterior probability, bootstrap and bootstrap respectively. Taken from Vlachakis, et al 2013. [14]

The classification of viruses in the genus *Flavivirus* is complicated by the extensive geographic distribution of the viruses and the variety of the arthropod vectors or vertebrate hosts associated with their respective transmission cycles. Evolutionary relationships of the flaviviruses have been revealed by phylogenetic analyses of the nucleotide sequences of a flavivirus 1 kb portion of the non-structural 5 (NS5) gene.[11, 15, 16] These analyses suggest that a primordial *flavivirus* gave origin to the non-vector and vector-borne clusters: the vector-borne cluster later branched into the insect-only, tick and mosquito clusters (Figure 2). The non-vector cluster is divided into three clades, the first two are rodent-

associated and the third is bat-associated. The cluster of mosquito-borne viruses contains nine clades; DEN belongs to clade IX.[13, 16] The cluster of mosquito-borne viruses has 22 viruses that are capable of causing illness in humans. There are 13 tick-borne viruses that can cause human illness, and 5 viruses with no known vector (NKV) that can cause human disease.(Figure 2)[17]

I.B.3. DENV Evolution and Epidemiology

I.B.3.a. DENV evolution and molecular phylogenies. The first evidence of different DENV serotypes was found in serological studies using antibodies made by inoculating laboratory animals with serum from acutely ill patients.[18] In the 1980's, RNA fingerprinting of DENV revealed genetic variability between the respective serotypes. [19, 20] Subsequently direct nucleotide sequencing of specific genes and entire genomes revealed the evolutionary relationship between different DENV serotypes and genotypes. DENV is divided into four antigenically distinct serotypes, which can be grouped into subtypes based on genetic differences detected by sequencing (genotypes), or by RNase T1 oligonucleotide fingerprinting (topotypes). [21, 22]

RNA fingerprinting studies provided the first evidence to differentiate DENV of the same serotypes. This technique does not provide results that are directly comparable across strains. The resulting groupings are called topotypes. The classification of DENV into genotypes is constantly improving as methods for sequencing are being developed. There is no uniform approach to determine which sequences will be compared among strains; also, there are sequences that contain errors (from sequencing or editing artifacts) which could lead to mistakes in interpretation. Also a bias exists because samples are commonly obtained from patients who are ill and likely with more severe disease and not enough samples are analyzed from mosquitoes, for this reason, quantitation of natural DENV diversity is premature.[23]

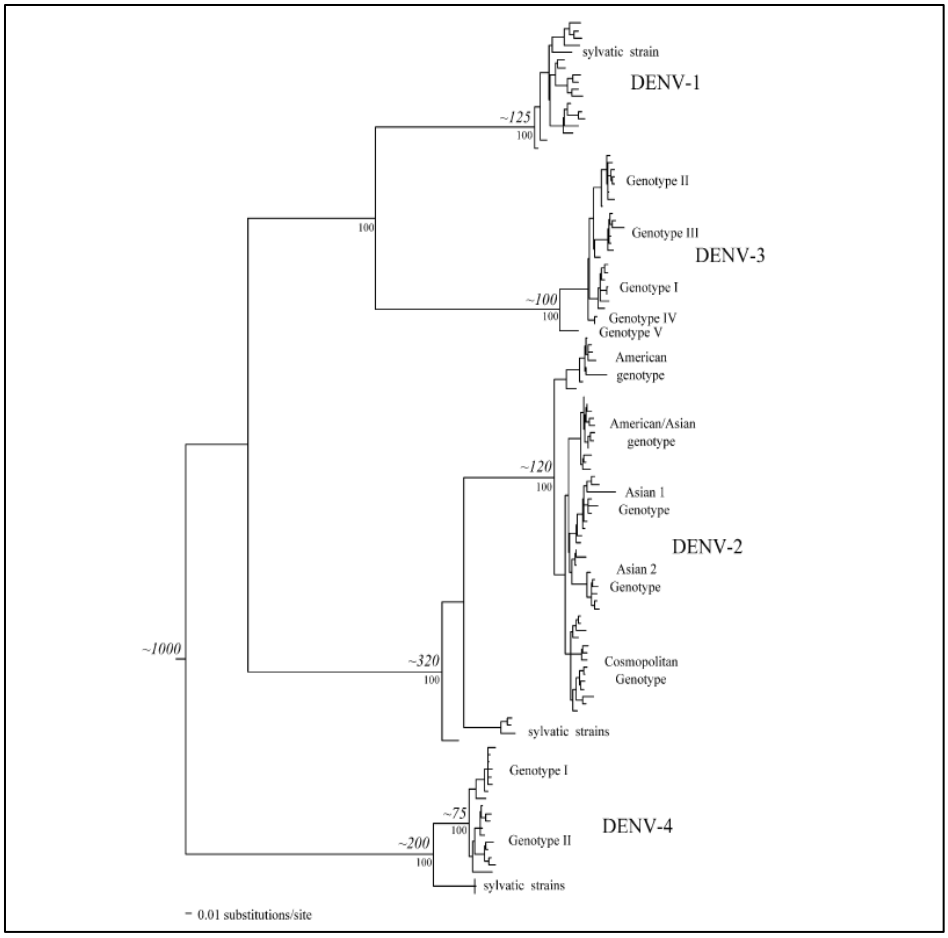


Figure 3. DENV phylogenetic tree illustrating the four serotypes and the genomes within.
Taken from Holmes, et al. 2003.[24]

The domains or genome regions selected for sequence comparison determines the analysis outcome and limits of interpretation, when long sequences are used, the trees generated from different genome regions usually correspond or overlap. Differences occur only when trying to interpret the minor branches of the trees. It is important to note that in those cases, arbitrary cutoff for genotypes and topotypes apply only to the region or sequences being compared. [22, 23] Complete genome sequences of DENV will allow establishing the natural, full range of nucleotide or amino acid variability within serotypes, genotypes and topotypes.[23]

The first divergence within DENV serotypes was found for DENV-4, and then DENV-2, and DENV-1 and -3 followed. The highest divergence was over 240 nts of the E/NS1 protein. [22]

The identification of genotypic lineages with different phenotypic characteristics has increased dramatically in recent years; phylogenetic analyses have revealed lineages with potentially increased virulence or transmissibility, changes in replication efficiency in vectors or vertebrate hosts, the potential to adapt to alternate vectors, etc. (Figure 3.) The ability to detect a particular genotype in a community may identify risk factors associated with transmission of that strain.[25, 26] These genotypic lineages with their concomitant phenotypic changes can be major factors in the changing epidemiology of DENV.

Phylogeny of DENV 1: Studies on the E gene (envelope protein) sequences have identified five DENV-1 genotypes based upon a cutoff point of 6% divergence for the respective genotypes: Genotype I: strains from Southeast Asia, China and East Africa; Genotype II: strains from Thailand collected in 1950-60; Genotype III: strains collected in Malaysia; Genotype IV: strains from the West Pacific islands and Australia; Genotype V: all strains collected in the Americas, some strains from West Africa and selected strains collected from Asia. The sylvatic strain from Malaysia apparently evolved earlier from a hypothetical ancestor virus shared by all DENV, which is the reason why most sylvatic viruses are basal (from earlier branches in the bootstrap) in all trees. DENV-1 has 8 topotypes.[22, 23, 27]

Phylogeny of DENV 2: The E/NS1 (envelope/non-structural protein 1) gene junction of 240 nucleotides was chosen for sequence analyses, and has revealed six genotypes of DENV-2. The genotypes are: Asian I, Asian II, Cosmopolitan, American, American/Asian and Sylvatic. Comparisons of DENV-2 E glycoprotein sequence revealed base substitutions in the entire gene accounting for up to 22% sequences divergence between genotypes. A comparison of aligned E glycoprotein amino acid sequences revealed the viruses differed by 10%. RNAse T1 oligonucleotide fingerprinting of DENV-2 isolates identified 10 topotypes. [22, 28, 29]

Phylogeny of DENV 3: Comparison of E gene sequence analysis identified four DENV-3 genotypes. The genotypes can be identified by geographical origin as follows: Genotype I: strains from

Indonesia, Malaysia, Philippines and recent isolates from South Pacific islands; Genotype II: strains from Thailand, Vietnam and Bangladesh; Genotype III: strains from Sri Lanka, India, Africa, Samoa and Thailand; Genotype IV: strains from Puerto Rico, Latin and Central America and the 1965 Tahiti strain. Sylvatic strains have not been isolated but their existence has been postulated because of the sero-conversion of sentinel monkeys in Malaysia. RNAse T1 oligonucleotide fingerprinting revealed five DENV-3 topotypes. [23, 27, 30]

Phylogeny of DENV 4: Analysis of the complete E gene sequences has revealed four DENV-4 genotypes. These genotypes are: Genotype I: strains from Thailand, the Philippines, Sri Lanka, and Japan; Genotype II: strains from Indonesia, Malaysia, Tahiti, the Caribbean and the Americas; Genotype III: Thai strains different from genotype 1 Thai isolates; Genotype IV: sylvatic strains from Malaysia. RNAseT1 oligonucleotide fingerprinting revealed five topotypes. [23, 27, 31]

I.B.3.b. Molecular epidemiology. DENV diverged from other *flaviviruses* about 2,000 years ago, providing ample time for evolutionary events to occur. However, it was only about 200 years ago that the rapid increase in viral genetic diversity began, coinciding with increased and unplanned urbanization, of mostly poor populations lacking basic health services. The emergence of DEN as a major public health problem in the past five decades is associated with the generation of substantial genetic variation within the viruses in the four DENV serotypes, and the phenotypic changes in the evolving genotypes. Approximately 390 million DENV infections occur per year, [32] and concomitantly infections with different DENV serotypes and genotypes are occurring at a higher frequency than ever before. Thus the potential for intramolecular evolutionary events to occur is greatly amplified and cause for concern because of the possibilities for viruses to evolve new phenotypic characteristics, such as increased virulence and transmissibility.[33] For example, introduction of Asian genotypes of DENV-2 into Latin America was associated with a dramatic increase in cases of DHF/DSS and the sweep of the new genotypes throughout the region.[34] Indeed the new genotypes have apparently supplanted the old

American genotype viruses in the region. What genotypic and phenotypic changes conditioned these sweeps and resulted in such dramatic changes in the epidemiology of DENV have been the subject of much speculation and investigation.[22, 35] Clearly, understanding the molecular epidemiology of the viruses is critical for developing effective surveillance and control programs.

Our understanding of the epidemic potential, virulence, pathogenicity, trafficking, and evolution of DENVs has benefited enormously from the application of molecular epidemiological techniques. Phylogenetic analyses and the resulting trees have an enormous value for the understanding of the epidemiology of the viruses when the branches are correlated with variables such as geographical location of the pathogen strains, the host in which they were identified, and virulence, among others. This type of analysis has been invaluable in understanding the geographical structuring of DENVs in nature, the evolutionary determinants of the gene flow, and the epidemic potential of the viruses.[33]

Associated with the introduction of new viruses/genotypes into new regions is the emergence of hyperendemicity of DENV in much of the tropical world. Hyperendemicity, with abundant vector populations and co-circulation of multiple DENV serotypes, promotes virus transmission and evolution.

Epidemics frequently have two or even more DENV serotypes co-circulating; the frequency of this phenomenon is increasing, and the circulation of the four DENV serotypes in the same community has become frequent in Southeast Asia, the Caribbean islands, Mexico and Central and South America, this is due to demographic and societal changes which have led to expansion in the geographic distribution of the mosquito vectors and the four DENV serotypes. This results in the co-circulation of multiple serotypes in a city or country (hyperendemicity), which is the single most common factor associated with the emergence of severe disease in a country. [36] Frequency of concurrent infections is variable from epidemic to epidemic; when more than one serotype is circulating the possibilities for dual infections of hosts are increased. Whether or not this contributes to increased severity of infections

remains to be determined, but dual infections of humans and mosquitoes likely contribute to the evolutionary and thus epidemic potential of the viruses.[36]

DENV-2 is the serotype most commonly associated with epidemics, and introduction of Asian genotypes of DENV-2 into Latin America was associated with the widespread emergence of epidemic DHF/DSS. [23] Southeast Asian genotype virus infection results in higher viremia titer than infection with American genotype virus, and likely is associated with the more severe disease outcome with infection with the former. Major differences between the two genotypes include nucleotide sequences and folding patterns of the 5' and 3' untranslated regions of the viral genome,[37] and amino acid charge differences in the prM, E, NS4B and NS5 proteins. [22, 23]

I.C. DENGUE VIRUS (DENV).

I.C.1. DENV molecular virology and replication. Because of the medical and veterinary importance of members of the *Flavivirus* genus, much is known about the structure and functions of the virion, the genome, and gene products. A brief summary follows:

I.C.1.a. Virion properties: Consistent with other viruses of this genus, DENV virions have a diameter of approximately 50 nm and have a single stranded positive sense RNA genome. The nucleocapsid has a spherical shape, is approximately 30 nm in diameter, and is surrounded by a lipid envelope. The calculated density of the virion is 1.23 g/cm^3, and the virion surface consists of 180 copies each of the E and M proteins inserted into the virus lipid envelope.[38-41]

I.C.1.b. Genome: The *Flavivirus* RNA genome is approximately 10.7 kb. [39, 42] The genome contains a single open reading frame (ORF). After virus entry and uncoating, the viral genome provides the template for translating the single ORF. The genome is expressed as a large polyprotein, which is cleaved into three structural proteins: C, prM and E, and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, by virus and host proteases (Figure 4). The 5' untranslated region (UTR) is short with only 100 nucleotides (nt), and has a type I cap structure. The 3' UTR of approximately

450 nt contains several conserved RNA structures but does not contain a terminal polyadenylate tract. Polyprotein synthesis occurs in the rough endoplasmic reticulum and signal peptides and membrane anchor regions direct the topology of the polyprotein with the structural proteins prM, E and the NS1 projecting to the lumen of the endoplasmic reticulum. This is also where virion assembly and maturation occur. [43]

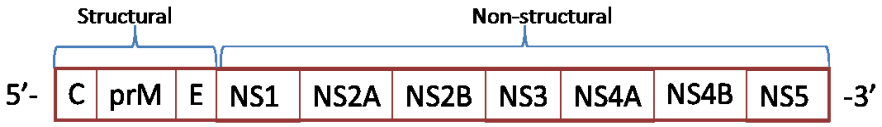


Figure 4. DENV genome and location of structural and nonstructural genes. Figure illustrates each of the genes contained in DENV genome. Abbreviations of the proteins: C capsid, prM pre-membrane, E envelope, NS non-structural.

I.C.1.c. Gene products. A brief description of DENV proteins follows:

The capsid (C) protein is the first protein to be synthesized during translation; it ranges from 9-12 kDa and contains 112-127 amino acids. Its charge is highly positive because of large numbers of lysine and arginine residues, which allows it to interact with the virion RNA. The C protein lacks an N-terminal, hydrophobic signal sequence, suggesting that C protein synthesis occurs on non-membrane bound ribosomes. The C-terminus acts as the transmembrane signal for the adjacent prM protein anchoring the C protein to a membrane at the replication site after cleavage at the N-terminus of prM protein. The C protein functions to gather the viral RNA into a nucleocapsid that forms the core of a virus particle. Although virus replication occurs in the cytoplasm, C protein can be found in the nucleus during infection. [39, 44, 45]

The membrane protein (M) is a glycoprotein containing two transmembrane helices. After it is cleaved during the maturation of the virion; it yields the pr peptide and the M protein. The prM protein has a role in controlling the fusion activity of the virion and acts as a chaperone by preventing the E protein from unfolding prior to virion maturation. prM (22 kDa) exists in the intracellular immature

virions, and M protein (8 kDa) is contained in extracellular mature virions. M is derived from prM by proteolytic cleavage during release of virus from cells. Proteolytic cleavage of a glycosylated prM precursor during the maturation of the virus in the acidic post-Golgi vesicles results in the M protein, which precedes the virus release from the cell. The formation of M is the terminal event in the morphogenesis of the virion, resulting in a large increase in virus infectivity and reorganization of the virus surface structure. [39, 43, 46]

The envelope (E) glycoprotein is a 51-60kDa protein that appears as a homodimer on the surface of mature virions, and it is associated with receptor binding, membrane fusion, hemagglutination of erythrocytes, and the induction of neutralizing antibodies in DEN patients, which could cause detectable changes in metabolic studies. The molecular conformation of E is dependent on the pH. At a neutral pH, E protein molecules have a flat dimeric conformation in a herringbone pattern on the virus surface. At acidic pH in the endosomal compartment, the molecules of the E protein protrude from the virus surface during the process of viral envelope and endosomal membrane fusion, facilitating the release of the viral genome into the cytoplasm of the cell. [38, 39, 47] E glycoprotein is widely exposed on the DENV virion. Immunity to the virus is mediated primarily by neutralizing antibodies to the E glycoprotein. It is this glycoprotein that determines each of the four DENV serotypes. The ectodomain of the E monomer folds largely into ß-sheets. It has three functional domains: DI, DII, and DIII. DI comprises the central region. DII functions in dimerization and contains a highly conserved, elongated structure that bears a fusion loop at its tip with a hydrophobic sequence conserved in flaviviruses that catalyzes type II fusion of the virus envelope with acidic endosomal membranes. DIII has an immunoglobulin-like fold and is involved in receptor binding. [38, 45, 47]

E protein induces the predominant antibody response during DENV infection for being the major protein exposed on the virus surface; this protein binds cellular receptors and fuses with host cell membranes. Neutralizing antibodies directed against the E protein are the principal mediators of

protection against DENV infection; some E antibodies are cross-reactive with plasminogen, which could increase the manifestations of severe DEN infection [38] that can be detectable in metabolomics studies.

The NS1 protein is a 46-55 kDa highly conserved nonstructural glycoprotein with two N-linked glycans. NS1 contains two signals of the type Asn-X-Thr used for the addition of the N-linked glycans, which occur in all flaviviruses. NS1 can be found in a soluble state; after the proteolytic cleavage of the viral polyprotein NS1 is found in the lumenal side of the endoplasmic reticulum.[48, 49]

NS1 is also associated with the virus RNA replication complex and participates in viral RNA replication, virion assembly and maturation.[50-52] NS1 exists in multiple forms in different compartments of virus-infected cells, including on the cell surface, from which it can be released into the extracellular compartments. After release, NS1 is found in the form of hexamers. [53, 54] NS1 is positioned in the lumen of the ER by a signal sequence corresponding to the final 24 amino acids of E. Then it is released from E at its amino terminus via cleavage by the ER resident host signal peptidase, and then the NS1 protein gets cleaved at its C-terminus from the downstream NS2A by a protease from the host cell. [38, 55]

In DENV secondary infections, a memory response of IgM and IgG antibodies to NS1 during the acute phase of the disease could cause the formation of immune complexes that can trigger inflammatory processes. For example, the activation of complement, which can cause endothelial cell retraction and increase vascular permeability, could cause the severe symptoms found in DHF/DSS.[55] Some NS1 antibodies are cross-reactive with human platelets and endothelial cells and may contribute to the severe disease manifestations.[38]

The NS2 proteins are classified as NS2A and NS2B. NS2A is a hydrophobic protein of ~20kDa molecular weight with several transmembrane domains required for proper proteolytic processing of the C terminus of NS1. NS2B is a hydrophobic protein of ~14.5kDa, but its function is not clear yet.

NS2A has been shown to contain a hydrophobic region that could act as a signal sequence for GPI (glycosyl-phosphatidylinositol) anchor addition. GPI is normally anchored to NS1 and plays a role in the polyprotein cleavage.[55]

The NS3 protein is hydrophilic protein of ~70kDa that functions as a chymotrypsin-like serine protease as well as a RNA helicase and RTPase/NTPase. The C terminal region of this protein resembles the sequence of nucleoside triphosphate-binding proteins involved in nucleic acid replication. NS3 is responsible for fatty acid synthase (FASN) recruitment to sites of DENV replication. NS3 protease domain is involved with FASN complex formation. During infection there is an elevation in the rate of fatty acid biosynthesis; *de novo* synthesized lipids co-fractionate with DENV RNA. NS3 stimulates the activity of FASN in vitro, enhancing the fatty acid biosynthesis to establish virus replication complexes. These changes can be detected in metabolic studies. [9, 56] This is relevant to the results presented in this dissertation because lipids are signaling mediators of regulatory events in the cell. Viruses as parasites rely on the host to fulfill their lipid requirements, which are essential for replication of DENV; the host derived membranes are rearranged to provide platforms for DENV replication factories.[57]

The NS4 proteins are hydrophobic proteins that can be classified as NS4A and NS4B. NS4A is a 16kDa protein that interacts with NS1 and becomes critical for replicase activity and has a role in membrane modification and replication. NS4B is a 27kDa protein that interacts with NS3, displacing it from ssRNA and has an important role in replication and in interferon (IFN) inhibition. [58] NS4A protein plays a role in DENV replication altering the intracellular host membranes. NSA4 resides primarily in ER-derived cytoplasmic dot-like structures that contain dsRNA and other DENV proteins. Thus, it can be concluded that NS4A is a component of the membrane-bound replication complex and is able to induce DENV-induced alterations in membranes of infected cells.[59] This could play an important role disturbing the homeostasis and metabolism of DENV-infected cells that can be detectable in metabolism studies.

The NS5 protein is a large protein of 105kDa with a Gly-Asp-Asp sequence that is common to viral RNA polymerases; it is a multifunctional RNA-dependent RNA polymerase. It is involved in viral RNA capping and is essential for virus replication. [39, 45] NS5 is one of the predominant DENV antigens recognized by antibodies following primary virus infection, and this immune response is broadened during secondary infection. This protein has the ability to induce IL-8 secretion *in vitro* which can contribute to inflammation and disease pathogenesis.[60]

I.C.1.d. DENV replication. DENV penetrates the cell by attachment to a receptor on the plasma membrane; DC-SIGN (dendritic cell specific ICAM-grabbing non-integrin), GRP78/BiP (glucose-regulating protein 78) and CD14-associated molecules have been suggested as potential primary receptors for DENV, nonetheless, the receptor is still unknown.[43] The mannose-specific lectin DC-SIGN on dendritic cells has been proposed to be the protein that interacts with the carbohydrate residues on the E protein.[61, 62] This is followed by uptake via receptor-mediated-endocytosis with pH-dependent fusion of the viral envelope with the endocytic vesicle membrane. The acidic pH can catalyze a conformational change in the E protein, resulting in fusion of the envelope of the virion and with a pre-lysosomal endosome membrane. This allows the viral core to access the cytosol and replication to commence.[43]

After DENV is exposed to acidic pH in the endocytic vesicles, important structural changes occur at the surface of the virion, including trimerization of the E protein and rearrangement of DII, exposing the flavivirus-conserved fusion domain and fusion with the endosomal membrane. When uncoating is completed, replication continues with immediate translation of the uncoated viral genome.

Once the polyprotein is translated, it is processed and cleaved and the viral proteins are produced (C, prM and E, and seven NS proteins). E and prM are anchored in the rough ER membranes during their synthesis and later transported to the Golgi apparatus for further processing.[45] During DENV maturation furin cleaves the prM in the trans-Golgi network; furin is a protein that disrupts the

trimer and allows rearrangement of the E proteins into the dimeric, low-lying conformation characteristic of the mature particle.[38]

DENV RNA replication requires RNA-RNA mediated circularization of the genome, which involves a minimum of three sets of complementary RNA sequences on both ends of the viral genome.[63] The interaction that occurs between RNA strands of the 5' and 3' ends modulates RNA translation, replication and encapsidation. This interaction acts like a promoter for viral polymerase RNA synthesis.[64] Variability in the viral virulence and pathogenicity has been associated with changes in the UTR regions.[37]

Structures that tend to be conserved among all DENV are the stem loop of approximately 100 nucleotides located at the extreme end (3'SL); a conserved sequence of approximately 10 nucleotides that serves as the cyclization sequence that binds to a 5' complementary region, which contains the translation initiation codon and an adjacent stem loop, that serves as the viral RNA polymerase promoter.[21]

I.C.2. DENV – transmission cycles, virus-vector interactions, and vector competence. DENV is maintained in nature in two distinct types of arbovirus cycles involving different primate hosts and different mosquito vectors. In the sylvatic or forest cycle, DENV is transmitted between susceptible primates and forest-dwelling mosquitoes. In the peridomestic or urban cycles, DENV is transmitted between susceptible humans principally by *Aedes aegypti* or *Aedes albopictus.* [2]

The relationship between the sylvatic forest cycle and the transmission of viruses to humans is not entirely clear.[65] In the rain forests of Asia and Africa, canopy-dwelling *Aedes* mosquitoes and lower primates are involved in the enzootic transmission cycle of DENV.[66] The virus can be maintained in the wild by vertical transmission in vectors in sylvatic forest cycles.[67] These sylvatic cycles include different species of lower primates and at least three subgenera of mosquitoes of the genus *Aedes*:

Stegomyia, *Finlaya* and *Diceromyia. Aedes* (*Stegomyia*) vectors include *Ae. aegypti*, *Ae. albopictus, Ae. polynesiensis*, and other members of the *Ae. scutellaris* group. [68, 69]

As mentioned, *Ae. aegypti* is the principal vector of DENV in urban cycles. The mosquito originated in Central Africa, where it is a forest-dwelling mosquito and breeds in tree holes and other water containing sites. The ancestral form of the mosquito, *Ae. aegypti formosus*, can still be collected there. *Ae. aegypti aegypti* (hereinafter referred to as *Ae. aegypti*) originated from this area, adapted to living with humans in peri-urban and urban environments, and has disseminated throughout most of the tropical world.[68]

Ae. aegypti has been and is the cause of inestimable human morbidity and mortality; it is the principal vector of DENV, YFV, and Chikungunya virus. *Ae. aegypti* is an extraordinary vector. The females live in close association with humans. They lay their eggs in water-containing objects, such as discarded tires, flower pots, buckets, water barrels, etc., which are abundant around homes. The mosquito larvae develop, pupate, and adults emerge near their human hosts. The female is endophilic and feeds preferably on human hosts in homes or other indoor environments. It has the ability to feed multiple times on human hosts during a single gonotrophic cycle, which dramatically increases the transmission potential of viruses in nature.[70-72]

Intensive studies of *Ae. aegypti* in Merida, Mexico confirmed the endophilic and anthropophilic (meaning that prefers to rest indoors after feeding on a human) nature of the vector. Infected female mosquitoes were detected in homes and schools.[73, 74] In homes, mosquitoes were predominantly found in bedrooms, followed by living/dining rooms, kitchens, bathrooms and storage rooms through the whole year. A small proportion of uninfected mosquitoes could be detected in the vicinity of the house. In schools, the predominant locations for infected mosquitoes were classrooms, offices and bathrooms, indicating that schools were a risky environment for DENV exposure. [73, 74]

DENV infection of *Ae. aegypti* has been the subject of much investigation. In nature *Ae.aegypti* ingests

DENV when it blood feeds upon a viremic human. The virus must infect, replicate and disseminate from the vector midgut, infect the salivary glands of the vector, and then be expectorated in saliva in order to be horizontally transmitted. When all of these steps are achieved, this is called a productive virus infection. There are reports of vertical transmission of DENV from infected females to some of their progeny. Almost all of these reports are based upon detection of DENV RNA, not infectious virus, in progeny mosquitoes, which could represent non-productive vector infections.[75]

Following ingestion, the virus must infect and replicate in the midgut epithelial cells of the vector. If the virus cannot infect these cells, this is described as midgut infection barrier (MIB), which could be conditioned by lack of appropriate receptors, early control of the virus in the midgut epithelium by the vector innate immune response, or other undiscovered mechanisms. If DENV infects midgut epithelial cells, the virions must release the genome, which must harness host cell machinery for genome replication, transcription, and translation of virus proteins, followed by virion maturation (see above). Infectious virus must then disseminate from the midgut epithelial cells through the basal lamina into the hemocele to infect secondary target organs. This may not be the only route of dissemination from the midgut cells; DENV has been detected in trachea of mosquitoes servicing midgut epithelial cells.[71] The importance of this potential route of viral dissemination remains to be determined. However, if the virus does not disseminate from the midgut, this is a midgut escape barrier (MEB). If the virus then does not infect the salivary glands, this is called a salivary gland infection barrier. [75] As described below, *Ae. aegypti* mosquitoes differ dramatically in their susceptibility to DENV productive infection or their vector competence. This is of great epidemiological importance, and is conditioned in large part by the before mentioned barriers to DENV productive infection of the vector. Perhaps even more significantly DENVs of different genotypes differ dramatically in their ability to productively infect *Ae. aegypti*.[71] American and Asian genotype DENVs are equally capable of infecting *Ae. aegypti*

midgut cells following oral challenge; however Asian genotype viruses efficiently disseminated from the midgut cells but the American genotype virus did not.[76]

Another component of vector competence that is also of great epidemiological significance is the lenght of time before a vector becomes productively infected. The period of time from ingestion of the viremic blood meal until the female can transmit the virus horizontally to a new human host is termed the extrinsic incubation period (EIP). The typical EIP for DENV in *Ae. aegypti* is 10 to 14 days; [75, 77] however, an Asian genotype of DENV-2 was detectable in the salivary glands of *Ae. aegypti* mosquitoes from Chetumal (Quintana Roo, Yucatan, Mexico) after only 4 days EIP, which could greatly increase DENV transmission in nature.[71] Once a female is infected she is infected for life and can transmit the virus to everyone whom she feeds upon.

Genetic variability in *Ae. aegypti* vector competence for flaviviruses has been long recognized, beginning with the classical studies of vector competence of geographic strains of *Ae. aegypti* for YFV. [78, 79] Population genetic studies of *Ae. aegypti* revealed extensive structuring in Mexico, presumably resulting from extensive gene flow in some regions, e.g. the western coast of Mexico, and restricted gene flow in other areas, e.g. between the Caribbean coast in Quintana Roo and the Yucatan.[80] *Ae. aegypti* from these various populations were challenged with DENV, and extensive variability in vector competence metrics, e.g., MIB, MEB, and salivary gland infection rates were documented. [81] Typically mosquitoes from collections from the Yucatan Peninsula, e.g. Chetumal in Quintana Roo, were the most vector competent and those from collections in the parts of the Caribbean coast in Quintana Roo were poorly vector competent [75]. More detailed studies of gene flow and vector competence of *Ae. aegypti* from Veracruz State revealed that the Neovolcanic Axis is a barrier to gene flow in *Ae. aegypti* and that some of these populations differed dramatically in vector competence. [82]

I.C.3. DEN history. DEN is an old disease; its origin has been the subject of much speculation. The first descriptions of a DEN-like illness date from 265-240 AD and were found in a Chinese encyclopedia that

referred to the disease as “water poison” and associated the disease with flying insects. This ancient description supports the theory that DENV originated in Asia, and it was maintained in a forest cycle that involved primates and canopy-dwelling mosquitoes before emerging as a human pathogen. [68, 83, 84]

In 1779 and 1780, the first reports of a major epidemic of DEN-like illness occurred in Asia, Africa and North America: In 1779 simultaneous epidemics occurred in Jakarta and Egypt. In 1780 an epidemic occurred in Pennsylvania, USA. By the end of the 18th century, DF was found globally. Multiple epidemics were reported in the Americas, the Caribbean, Asia, Australia, and in the Pacific. [68, 85] Cases of DHF/DSS have been sporadically reported since 1780.[85] Many outbreaks of DF and DHF/SS have occurred worldwide since then.

During World War II, epidemic DF started to become common. DF and malaria caused havoc in both the Japanese and Allied forces, causing thousands of cases in soldiers. By the time the war finished, DENV was hyperendemic in many countries in Asia resulting in co-circulation of the four serotypes. The post-war economic growth in Asia and expanded commerce areas were the catalyst for both urban growth and DHF/DSS epidemics. By the 1980s, DHF was one of the leading causes of hospitalization and death among children in Southeast Asia.[3] DF and DHF/SS are now pandemic throughout the tropical world.

DENV was first isolated in the 1940s during WWII. In 1943, Hotta and Kimura in Japan isolated for the first time DENV by injecting serum from an acutely ill patient intracranially into a mouse; this was the Mochizuki strain, later classified as DENV-1. [86] This result was published in an obscure journal. At around the same time Sabin and his group isolated DENV by the same technique. They developed a hemagglutination inhibition test, which revealed serologic similarities between DENVs isolated in India, New Guinea and Hawaii. This Hawaiian isolate was considered the prototype virus (Haw-DENV-1) of DENV-1. Serologic studies demonstrated that DENV-1 was the predominant virus in the Philippines and Greece in the 1920’s, in the South Pacific in the 1930’s and in the Pacific and Asia during WWII, all during

a major DENV pandemic.[68] In New Guinea, Sabin described an antigenically different virus strain that was designated as DENV-2 (NG-C-DENV-2).[18, 87]

DENV-3 and 4 were subsequently isolated in Manila, Philippines from patients with severe disease during an epidemic in 1956.[88] Thousands of DENVs have been isolated in the tropics since the original isolates were described; all of these fit into the four-serotype classification. DENV-2 and DENV-3 have been associated with more severe disease and greater mortality through different epidemics.[77] Bancroft was the first to describe DENV transmission by its vector, *Ae. aegypti*, which was confirmed by Cleland and Bradley.[68]

I.C.4. Emergence of DHF/DSS in the Americas. In 1789 the first description of a DEN-like disease was reported in the Americas, and it was based on an epidemic that occurred in Philadelphia during the1780's.[85, 89] After that, in the 1800's four DEN epidemics affected the Caribbean Basin countries and the southern U.S. Several epidemics were reported in the Caribbean in the early 1900's. [68, 90]

DENV-2 was isolated in mice for the first time in the Americas in 1953 in Port of Spain, Trinidad from a patient with DF.[91] DENV-3 was reported for the first time in the Americas in 1963,and the virus was isolated in Puerto Rico during an epidemic in that same year.[92] DENV-1 was isolated for the first time in 1977-1980 in the Americas in Kingston, Jamaica.[92] DENV-4 caused epidemics in the Caribbean, South and Central America, and it was found to be the cause of severe disease in Yucatan in 1984.[93] Epidemic DHF/DSS was first reported in Cuba in 1981. There had been a large epidemic of DF following the introduction of DENV-1 in Cuba in 1977. DENV-2 was introduced in 1981 and a large epidemic of DHF/DSS occurred, which was likely associated with secondary infection of patients previously infected with DENV-1.[92]

In Mexico, the first case of DF was reported in Chiapas in 1978; a similar case was reported in the Yucatan for the first time in 1979. DF outbreaks occurred in most of the states of Mexico and the southern U.S. by 1980.[93, 94] In the spring of 1980, DENV-1 was isolated for the first time in Merida,

Mexico; in 1984 an outbreak of DENV- 4 occurred in the Yucatan and 9 cases of severe hemorrhagic disease were reported although just one case met the WHO criteria to be classified as DHF. [93] Many DEN epidemics have been documented in Yucatan since then: DENV-1 in 1979-1982, DENV-1 and 4 in 1984, DENV-2 and 4 in 1991, DENV-1, 2 and 4 in 1994, and all serotypes in 1995-1997. By 1985, 72.5% of participants in a serosurvey had antibodies to DENV. The co-circulation of serotypes favored the increase in DHF/DSS cases starting in 1995 with the introduction of DENV-3.[35] In 2001-2002 an epidemic occurred and several cases presented hemorrhagic manifestations.[95] After a period with low incidence of cases, DHF cases increased again in 2002 and more than 1,000 cases per year have been reported since that year in Yucatan.[96]

Currently, the Americas contribute up to 14% of the total of DEN cases estimated in the world, with most of the cases reported from Brazil and Mexico.[32]

I.C.5. DEN disease burden. Three billion people worldwide are at risk for DENV infection in tropical and subtropical climates. DENV transmission is ubiquitous through the tropics with the highest risk in the Americas and Asia (Figure 5). [32, 41, 97]

Since the 1950's the incidence of DHF/DSS has increased over 500-fold and more than 100 countries have been affected by outbreaks. [41, 98] Approximately 50,000 hospitalizations per year are caused by DHF/DSS; the case fatality rate in some areas is up to 5%.[32, 41]

WHO estimates that approximately 100 million DENV infections occur annually, and it estimates that approximately 3% of those affected with DHF/DSS die. However a recent report estimates that 390 million DENV infections occur worldwide each year. In 2010, 96 million infections were reported to public health authorities. An additional 294 million infections are estimated to have occurred in that same year, but were not reported in public health surveillance systems, likely because they were mild or asymptomatic infections. The estimated 390 million DENV infections occurring annually would dramatically increase the amount of virus circulating in nature, and thus may be of major

epidemiological significance because of the likelihood of dramatic increase in secondary infections leading to more severe DEN disease outcomes. Clearly, the numbers of DENV infections have been underestimated. According to a recent report, only 10% of symptomatic DEN cases are communicated to health authorities.[41] Likely the most important cause of this disparity between DENV infections and reported cases is the low proportion of infected individuals seeking care in formal health facilities.[32]

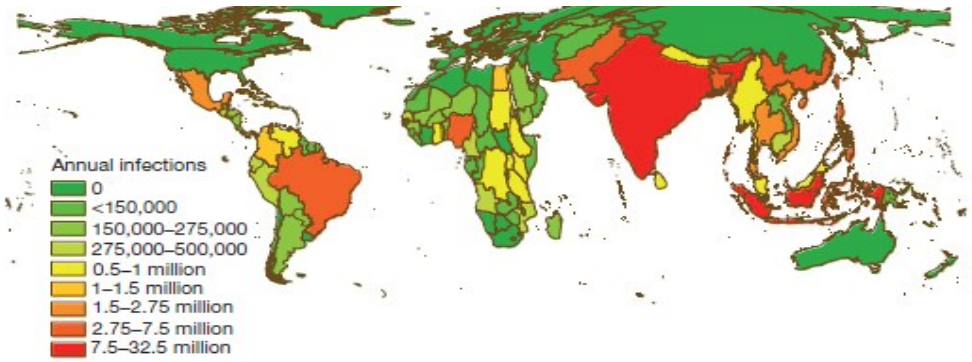


Figure 5. Estimated DENV infections throughout the world in 2010. Red colored countries have a higher incidence of DENV infections than green colored countries. Taken from Bhatt et al 2013. [32]

DEN incidence has increased dramatically in the last five decades. The causes of this increase are multifactorial and include population growth, unplanned urbanization in tropical and subtropical countries, the lack of successful vector control, lack of a vaccine, public health policies, and international travel.[99]

The burden of disease has shown important variation in geographic and temporal incidence of cases.[41] Primary DENV infections are common and frequently asymptomatic in younger children; symptomatic DEN is more commonly diagnosed in school-aged children or young adults. Approximately 22-292 per 1,000 children are infected each year in Asian countries and 1-8 per 1,000 children are admitted to hospital per year. Children with DEN infections are more susceptible to shock, and infections in infants have a higher mortality rate, as high as 3-4% in hospital admitted cases.[41, 100]

I.C.6. DEN pathogenesis and disease in humans. The pathogenic mechanisms by which the four different DENV serotypes (DENV 1-4) condition disease outcomes (e.g., asymptomatic infection, DF, or

DHF/DSS) remain the subject of much investigation. DF is defined as a self-limiting, influenza-like disease that can occur concurrently with retro-ocular pain, rash, arthralgias and myalgias. In laboratory analysis of DF patients, it is common to find leukopenia, thrombocytopenia and mild elevations in hepatic transaminases in serum.[101] Patients usually recover without sequelae, except for fatigue that could last for several weeks.

The most severe form of DEN disease is DHF/DSS. Common findings are headaches, fever, myalgias, liver involvement, and vascular permeability defect resulting in plasma leakage, multifactorial hemostatic abnormalities, such as marked thrombocytopenia, and bleeding diathesis.[102] These factors can lead to both internal and external bleeding and vascular leakage that can cause shock and death in approximately 3-5% of the cases.

Vascular leakage is caused by a diffuse increase in capillary permeability, and manifests with hemoconcentration, pleural effusion and/or ascites. The bleeding tendency is due to both capillary fragility and marked thrombocytopenia. Bleeding manifestations may fall into a broad spectrum such as a positive tourniquet test to severe gastrointestinal bleeding that causes shock and death.[103] It has been postulated that plasma leakage is caused by functional (caused by the effects of complement activation, cytokines and immunoglobulins on the endothelial cells of capillaries) and not by anatomical disturbances of capillary endothelial cells; the evidence supporting this is based on the successful outcome of the cases who receive appropriate fluid resuscitation and in histopathology studies of blood vessels that showed no evidence of endothelial cell destruction.

Host factors play a very important role in the pathogenesis of DHF; non-associated conditions present at the time of DENV infection can also exacerbate the severity of the disease, such as age, race/ethnicity, pregnancy, nutritional state, chronic diseases like asthma, coagulopathies, and diabetes, among others. [70, 104] It has been postulated that another factor that could influence the occurrence of a severe DEN outcome includes mutations in the vitamin D receptor (VDR), since activated B and T

lymphocytes express VDR and 1, 25 vitamin D3 affects monocytes, which have important roles for DENV infection and replication.[105, 106] This factor will be reviewed with more detail in Chapter 4.

Two major hypotheses have been proposed to explain the progression of the patient to DHF/DSS: 1) infection with virulent virus strains [34, 107] or 2) antibody dependent enhancement of infection[108]. Both hypotheses are supported by epidemiological data. The first hypothesis postulates that severe disease or DHF/DSS can be associated with specific outbreaks caused by a more virulent virus strain. [107] In 1972, during an epidemic in Niue Island, hemorrhagic manifestations were observed in patients with primary DENV infections. Serologic analysis confirmed cases by a fourfold or greater rise in titer of hemagglutination inhibition and neutralizing DENV antibodies. The findings showed that the patients under 25 years had low titers of DENV-2 neutralizing antibody, and very few patients had neutralizing antibody against the other DENV serotypes. The persons who were 30 years or older likely experienced a secondary infection. It was noted that clinical manifestations were similar (hemorrhage, shock) in persons experiencing primary and secondary infections.[107] This evidence supports the first theory.

Potential viral molecular determinants of severe disease have been identified: 1) amino acid 390 of the E protein that has the ability to alter virion binding to host cells, 2) the 5'NTR at the downstream loop that could be involved in translation initiation and 3) the 3'NTR at the upstream 300 nt of the 3' NTR, that has the ability to regulate the viral replication by the formation of replicative intermediates, but no one site could be specifically correlated with attenuation or severe disease in humans.[37, 107, 109]

The second hypothesis postulates that severe disease could be conditioned by antibody dependent enhancement (ADE) occurring during sequential infections.[108, 110] This will be covered in more detail in further sections.

I.C.6.a. DENV tropisms and innate and acquired immune response. Although DENV can be transmitted iatrogenically (e.g. blood transfusion), the vast majority of infections result from the bite of mosquito vectors. When the mosquito bites, DENV is injected to the skin and tissues surrounding the bite site. Initial target cells include Langerhans cells or epidermal dendritic cells (DC) that express Langerin, which is a C-type lectin. DENV infection of these cells is likely mediated via the non-specific lectin receptor dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN).[111, 112]

i. Innate immune response. The innate immune response is the first and immediate line of defense to DENV infection. This response includes the rapid recognition of pathogen-associated molecular patterns (PAMPs), by monocytes or macrophages, DCs, and natural killer (NK) cells. [113] Monocytes and tissue macrophages have been reported to be the principal target or most permissive cell population for DENV infection *in vitro*. [103] Immature DCs are sentinels and play a role in the early control of DENV infection; these comprise four cell types: CD14+blood, monocyte-derived DCs (moDCs), CD34+ hematopoietic progenitor cell (HPC)-derived dermal or interstitial DCs (DDC-IDCs), CD34+HPC-derived Langerhans cells (LCs) and plasmacytoid DCs.[61, 114] The LCs and DDC-IDCs carry typical myeloid-type DC markers and the LCs also express Langerin. Plasmacytoid DCs (PDCs) and blood-precursor myeloid DCs (pre-moDCs) have an important role in antiviral innate immunity and shaping Th1 adaptive immune responses. DCs in the peripheral blood and lymphoid tissues were reported to make Type 1-IFN in response to microbial stimuli. PDCs are the most potent interferon (IFN) producing cells to viral pathogens (Figure 6). [114]

Plasmacytoid DCs and blood-precursor myeloid DCs shape Th1 adaptive immune responses. DCs in the peripheral blood and lymphoid tissues make type-I IFN αß in response to microbial stimuli.[114] After DENV infects a host, components of the innate immune response include type 1 IFNs, inflammatory cytokines, complement response, NK cell immunity, programmed cell death (apoptosis)

and autophagy.[113] In order for DENV to disseminate within a host, it must evade or modulate these innate immune response components.

Productive DENV infection in immature myeloid DC (DC1a+) will activate these cells. Activated DCs secrete TNF-α (tumor necrosis factor-alpha) and IFN-α. In addition DENV-stimulated DCs are able to express maturation markers such as HLA-DR (human leukocyte antigen complex), CD11b (cluster differentiation), and DC83. The IFN-α can enhance the activation of DENV infected DCs, as well as secreted cytokines like IFN-γ and TNF-α and low levels of IL-12. [114]

DENV is recognized within hours of infection by pathogen-recognition receptors (PRRs), potentially including toll-like receptors (TLR) 3 and 7, retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation associated gene 5 (MDA5).[115] PAMP recognition triggers the innate immunity against DENV, when dsRNA (the PAMP) is sensed by cytoplasmic RNA helicases RIG-I and MDA5, as well as by TLR-3. TLR-3 also senses viral nucleic acid and envelope glycoproteins in the extracellular and endosomal compartments.[116, 117] DExD/H box containing RNA helicases (RIG-I, MDA-5) recognize intracellular dsRNA. This is called the TLR- independent IFN induction pathway. RIG-I and MDA-5 contain caspase-recruiting domains (CARD), which help these molecules to interact with IFN promoter stimulated-1, mitochondrial antiviral signaling protein (MAVS) [117, 118]

TLR-3 and RNA helicases are triggered by dsRNA (Figure 6). The two parallel viral recognition pathways converge at the IFN regulatory factor-3 (IRF3). IRF-3 is postulated to start antiviral responses, like activation of type I IFN, ISGs and proinflammatory cytokines (Figure 6).[119] The molecular mechanism of TLR involves the TIR domain (Toll/IL-1 receptor-like), which is present in all TLR and IL-1R compounds, and initiates a signaling cascade through interactions with TIR domain-containing adapters (MyD88). After interaction with TLRs or IL-1Rs, MyD88 recruits members of the IRAK (interleukin-1 receptor-associated kinase) family through interactions between the death domains. Once

phosphorylated, IRAK1 and 4 dissociate from receptor complex and then associate with TRAF6, which induces the expression of inflammatory cytokines and the phosphorylation cascade (Figure 6).[114]

The activation of the transcription factors IRF-3, IRF-7 and NF-κß through the TLR or RIG-I/MDA5 pathways will result in the production of IFN-α/β which will bind to the IFN-α/β receptor in the surface of uninfected cells, activating the JAK/STAT signal transduction pathway (Janus kinase-JAK/Signal transducer and activator of transcription-STAT) (Figure 6). This will activate the expression of interferon-stimulated genes (ISGs), establishing antiviral, antiproliferative, and immunoregulatory states in host cells. DENV NS2A, NS4A and NS4B proteins are capable of impairing the JAK/STAT pathway by reduction of phosphorylation and translocation of STAT1, which is an important component of the type 1 IFN transduction pathway. NS5 is capable of blocking TyK2 phosphorylation and degrading STAT2.[113]

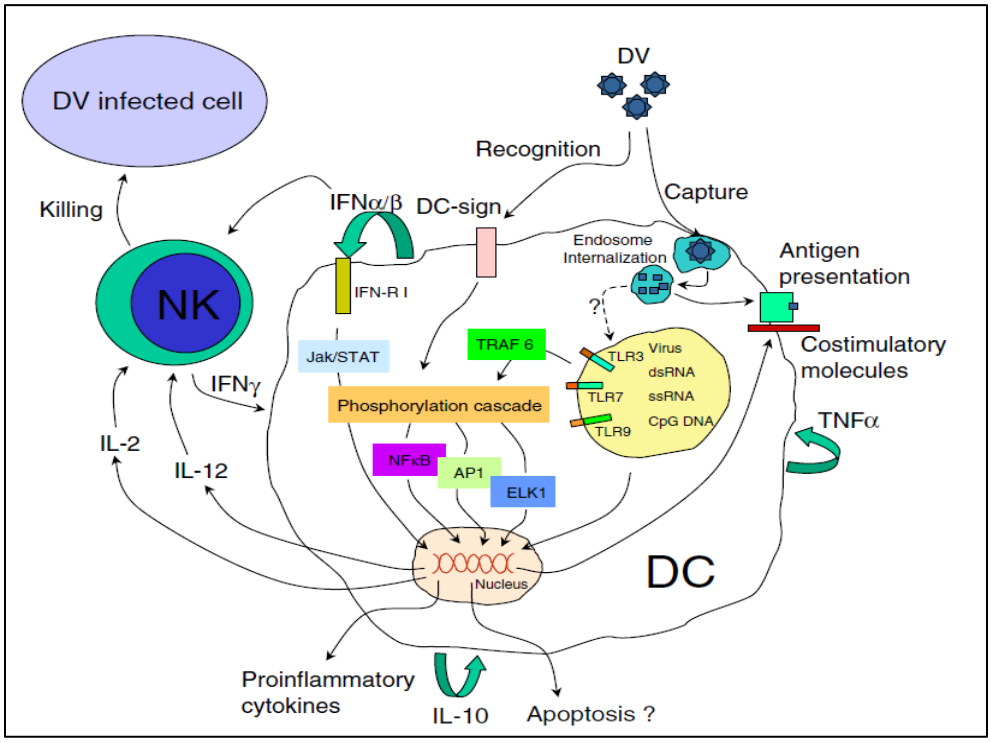


Figure 6. Innate immune response to DENV infection. DENV infected dendritic cells activate natural killer cells during the early phase of the innate immune response, which recognize and kill target cells that are coated with antibody or fail to express HMC class I proteins. Taken from Navarro-Sanchez, 2005.[114]

NK cells lyse DENV-infected cells by the release of cytotoxic granules containing perforin and granzymes or by engaging apoptosis-inducing receptors on infected cells. Major histocompatibility complex class I molecules (MHC) are able to inhibit some of the NK cytolytic activity by inhibitory receptors, including: the killer cell immunoglobulin (Ig)-like receptor (KIR) family, the Ig-like transcript/leukocyte Ig-like receptor (LIR) family and the type C-lectin family. It has been postulated that the C-type lectin domain family 5 member A (CLEC5A) directly interacts with the DENV virion, resulting in the release of inflammatory cytokines.[120] MHC-1 is increased approximately 96 hours after infection, theoretically by uncleaved C-prM protein. Caspases mediate apoptosis of infected cells, thereby stopping viral replication. Caspases are important components of the machinery responsible for apoptosis and are produced as catalytically inactive zymogens and activated through a proteolytic mechanism during apoptosis. There are effector caspases (such as caspase 3 or 7) that are activated through initiator caspases (caspase 9) through cleavage at specific internal Asp residues. The initiator caspases are autoactivated. The activation of procaspase 9 is facilitated by Apaf-1 and cytochrome C; these form a complex called apoptosome in the presence of dATP or ATP. [113, 121]

ii. Adaptive immune response. The adaptive immune response is important in the resolution of the infection and plays a key role in protection from reinfection, and it also can be responsible for the pathogenesis observed in severe DEN. Antibodies to the E protein can prevent infection by different mechanisms including 1) blocking of attachment, 2) inhibition of fusion of the virus membrane with the endocytic vacuole membrane that inhibits release of viral RNA in the cytoplasm, and 3) lysis of the antibody-coated infected cell by complement. After infection, CD4 and CD8-T cell responses specific for DENV are developed to multiple epitopes of DENV antigens. [38, 122] DENV reactive CD4+ and CD8+ T cells produce elevated levels of IFN-γ and TNF-α, TNF-ß and chemokines like macrophage inhibitory protein (MIP)-1ß after interaction with DENV-infected APCs, and are efficient at lysis of DENV infected cells in vitro. [104, 123]

The adaptive immune response has two principal components: 1) the humoral immune response, and 2) the cellular immune response. Both are critical in the response of the human host to DENV infection, and both will be reviewed briefly.

a) The humoral immune response influences infection and dissemination of DENV with the ability to neutralize or enhance DENV infection.[124] It has been postulated that a large fraction of DENV-specific serum antibody response in humans consists of cross-reactive, poorly neutralizing antibodies and a small fraction is serotype-specific, potent inhibitory antibodies.[125]

If the patient was not previously infected with a flavivirus or immunized with a flavivirus vaccine, and is experiencing a primary infection, anti-DENV IgM antibodies appear first and are present in almost every DEN patient generally by 5-8 days following the onset of symptoms. The levels of IgM will slowly decline and disappear after 2 to 3 months. IgG (IgG1 and IgG3 being the predominant subclasses), antibodies can be detected 7 days after of onset of symptoms. In antibody-dependent enhancement (ADE), IgG3 is the highest and IgG4 the lowest IgG isotype.[126] These levels will increase slowly and will be detectable after years and in most cases, detectable for life.[38] If the patient is experiencing a heterologous secondary DENV infection, the IgM response will be significantly lower than that resulting from a primary infection and may not even be detectable in some cases. In secondary infections, IgG will be present in high concentrations and will bind to newly produced virus that adheres to and is carried by mononuclear cells.[6, 38, 124]

The complement system is a component of humoral innate immunity and also a key modulator of adaptive immunity. It plays a role in the proper functioning of the hemostatic system for defense against pathogens. There are three different complement pathways, the classic, alternative and lectin, which result in the activation of opsonization, recruitment of phagocytes, and lysis of infected cells (Figure 7).

Mannose-binding lectin (MBL) binds specific mannose-containing glycans on the surfaces of DENV and initiates complement activation via the lectin pathway. In the alternative pathway, interactions among cell surface-bound C3b (complement component 3) and protease factor D, generate the C3bBb convertase that amplifies C3b production, which finally leads to the C5b-9 membrane attack complex and cell membrane damage (Figure 7). In the classical and lectin pathways, the binding of NS1 to C4 reduces C4b deposition and C3 convertase activity.[113]

In DEN pathogenesis (Figure 7) during the time of defervescence, (when plasma leakage could occur), abnormally high levels of C3a and C5a are detected in plasma, which is followed by an accelerated consumption and reduction of the complement components in patients with DSS. It has been postulated that DENV does not efficiently activate complement; DENV requires anti-NS1 antibodies for complement consumption and C5b-9 generation.[127]

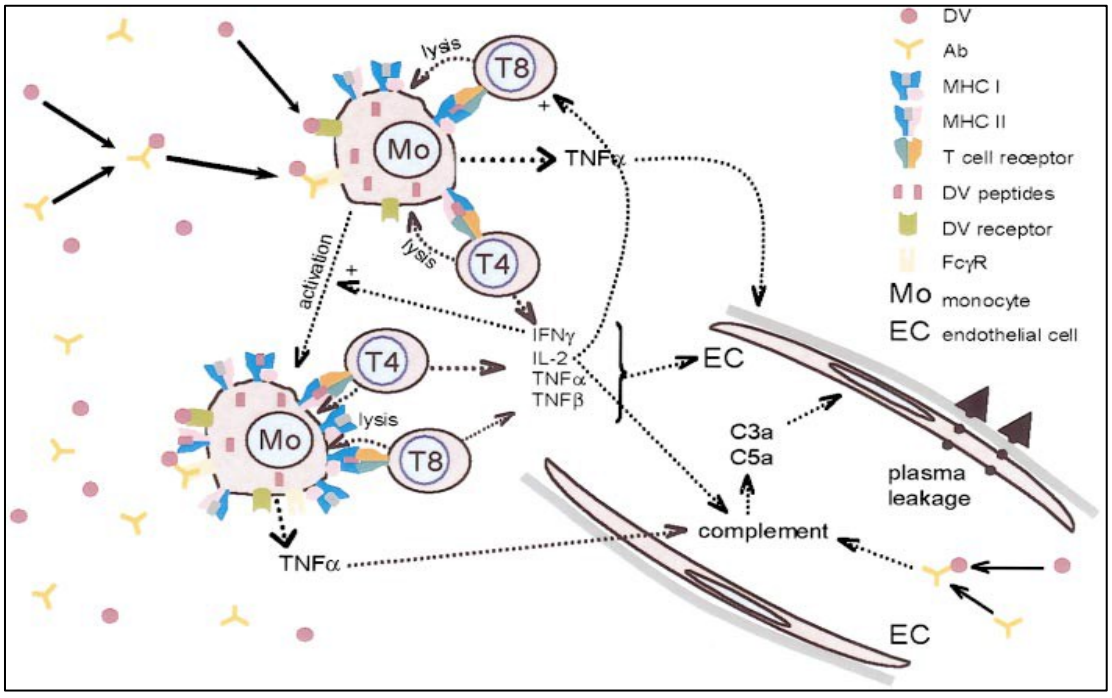


Figure 7. Model of immunopathogenesis of plasma leakage in DHF/DSS. Figure represents the immunopathogenesis process that occurs in secondary DENV infections that develop DHF/DSS. The serotype cross-reactive antibodies present at the time of secondary DENV infections, bind to virions without neutralization, this enhances the entry of DENV into monocytic cells that express immunoglobulin receptors. Serotype cross-reactive memory CD4 and CD8 T lymphocytes recognize viral

antigens by class I and II HLA molecules. T lymphocytes release cytokines such as IFN-γ, IL-2 and TNFα and β which lyse DENV-infected monocytes. These cytokines have direct effects on endothelial cells to induce plasma leakage. The complement cascade is activated by virus-antibody complexes as well as several cytokines to release C3a and C5a that also affect endothelial cells and vascular permeability. Taken from Rothman and Ennis, 1999.[103]

The activation of the membrane attack complex (C5b-C9 complex) of the complement system could activate production of inflammatory cytokines associated with DHF/DSS.[127, 128] Also, IgG1, IgG2, IgG3, and IgG4 are known to activate the classical complement pathway, although IgG1 and IgG3 are the most effective.[129]

It has been hypothesized that NS1 plays a role in the binding of heterotypic antibodies and complement activation by directly interacting with C4 which will lead to decreased activation of lectin and classical pathways. [112, 127] Some NS1 antibodies are cross-reactive with human platelets and endothelial cells, and some E antibodies are cross-reactive with plasminogen. These responses may contribute to the severe disease manifestations; however the mechanisms of activation of DEN pathogenesis remain to be fully determined.[38]

The E glycoprotein is a dominant target of the antibodies against DENV; these inhibit viral binding to cells and neutralize viral infectivity *in vitro*.[130] Neutralization occurs when the molecules of antibody bind to the accessible epitopes on the E protein and prevent binding to the target cell and the release of virion RNA into the cytoplasm of the host cell. [38, 41, 131]

The most effective neutralizing-antibodies are strain specific and recognize epitopes in receptor binding domain III (DIII) of the E protein. Cross reactive antibodies recognize domain II (E-DII), specifically the conserved fusion loop of E protein on the four DENV serotypes.[130] E-DIII-specific antibodies are able to create strong protective responses; this type of antibody is not immunodominant.[126] The functional properties of cross-reactive antibodies on cells lacking Fc receptors have shown that this type of antibody neutralizes virus infection by blocking binding of the

virus to the cellular receptor.[132] The complement system favors this mechanism by binding to complement-fixing antibodies resulting in neutralization of viral infection.[133]

b) Cellular immune response contributes to the clearance of an established infection. Cytotoxic T lymphocytes (CTLs) recognize viral peptides presented by MHC-1 molecules, eliminating cells producing abnormal or foreign proteins. DCs capture antigens and process them to become immunogenic peptides, and present peptides in the context of MHC-1 and 2 class molecules to T-cells.[114] DCs are also considered antigen presenting cells (APCs). After activation, DCs mature and migrate to the lymph nodes where monocytes and macrophages are also recruited, and become the targets for the infection. After infection, the virus is amplified and disseminated through the lymphatic system. In the lymph nodes the infected DCs will present antigens to naïve T-cells. This will cause proliferation and differentiation of T-cells to effector cells that will trigger adaptive immune responses. After DCs are activated, there will be up-regulation of MHC-1 and 2, and the release of proinflammatory cytokines (such as IFN-γ and TNF-α) and chemokines that will stimulate T lymphocytes. The release of these factors could play a crucial role in the early immune response that favors the extravasations of NK cells and DCs from the intravascular fluid.

The secretion of type 1 IFNs by DCs will contribute to the generation of innate and adaptive immune responses. DENV specific memory T cells have high binding affinity to DENV antigens from a primary infection and poor binding affinity to antigens from the secondary infection of heterologous serotypes; this phenomenon is called “original antigenic sin”.[134]

DENV infects different cells of the mononuclear lineage, for example, blood-derived monocytes, myeloid DCs, spleen and liver macrophages. Infected mononuclear cells will eventually die by apoptosis, while DCs will be stimulated to produce inflammatory mediators, monocytes will induce TNFα production. Primary human monocytes are able to activate endothelial cells, and this effect can be blocked by the addition of a monoclonal antibody to TNFα.[112, 135]

Transcription of RANTES (regulated on activation, normal T cell expressed and secreted or CCL5) and IL-8 (interleukin 8) are up-regulated during the infection of human endothelial cells with DENV. ICAM-1 expression has been found to be increased in patients in acute febrile phase.[136] ICAM-1 is an inflammatory mediator in DEN and has been associated with damage to endothelial cells. TNF-α has also been associated with damage to microvascular endothelium and endothelial cells.[136]

The number of target cells infected and the resulting viremia titers could determine the ratio of different proinflammatory and anti-inflammatory cytokines and chemokines and thus the intensity of the inflammatory response and effects on the hemostatic system. [112, 137] Increased levels of (anti-inflammatory cytokine) IL-10 have been associated with Th2 responses in antibody-dependent enhancement (ADE). In addition, IL-2 receptors, soluble CD8 TNF-α and IFN-γ (pro-inflammatory cytokines) have been found elevated in DHF/DSS. [104, 112]

iii. Secondary DENV infections. In secondary infections a different DENV serotype induces immune responses, giving the secondary infection a heterologous nature (Figure 7 and 8). Serotype cross-reactive T cell clones frequently recognize epitopes in the nonstructural (NS) proteins. NS3 is considered a major target for CD4+ and CD8+ lymphocytes. In a secondary DENV infection, the specific memory T lymphocyte population will be composed in its majority by serotype-cross reactive T lymphocyte clones with the ability of recognizing epitopes in NS proteins. This indicates that cross-reactive memory T lymphocytes induced by the primary DENV infection proliferate most rapidly in a secondary exposure to DENV.[103, 122]The antibodies and memory T cells induced by the primary infection will encounter antigens that differ in sequence from their original target antigen.[104] The sequence differences at the antibody-binding sites reduce the avidity of the interactions among the pre-existing antibodies and the new DENV serotype.

These interactions have a significant impact on the ability of the antibodies to neutralize virus infectivity, but there is still enough binding of antibody to the virion to trigger ADE. [103, 138] The

consequences of sequence differences in T cell epitopes is complex, due to the wide range of effector functions of T cells. Altered peptide ligands can induce different activation signals in antigen specific T lymphocytes, and with this modulate the specific effector functions of (cluster of differentiation) CD4+ and CD8+ T cells.[139]

The activation of DENV-specific T lymphocytes during interactions with DENV infected monocytes could cause increased capillary permeability by cytokine production by activated DENV-specific CD4+ T lymphocytes. These produce high levels of IFN-γ, which is also produced by CD8+ T lymphocytes. These cytokines may induce capillary leakage through multiple direct and indirect effects in the vascular endothelium. [103, 140]

The reactivation of cross-reactive memory T cells specific to the previous rather than the current infecting DENV will cause a higher viremia titer, a longer viral clearance time and a greater secretion of cytokines along with increased apoptosis of infected and bystander cells. Cytokines play a key role in the immunopathogenesis of DEN; their proinflammatory effect presumably causes damage to endothelial cells leading to leaky junctions and increased capillary permeability in severe disease (Figure 8). Target cell lysis is an immunopathologic mechanism of activated DENV specific T-lymphocytes. In this mechanism; CD4+ and CD8+ lymphocytes lyse DENV infected target cells *in vitro* by a perforin-dependent mechanism.[103]

It has been hypothesized that plasma leakage in secondary DENV infections is due to the synergistic effects of IFN-γ and IFN-α and activated complement proteins on endothelial cells throughout the body.[103]

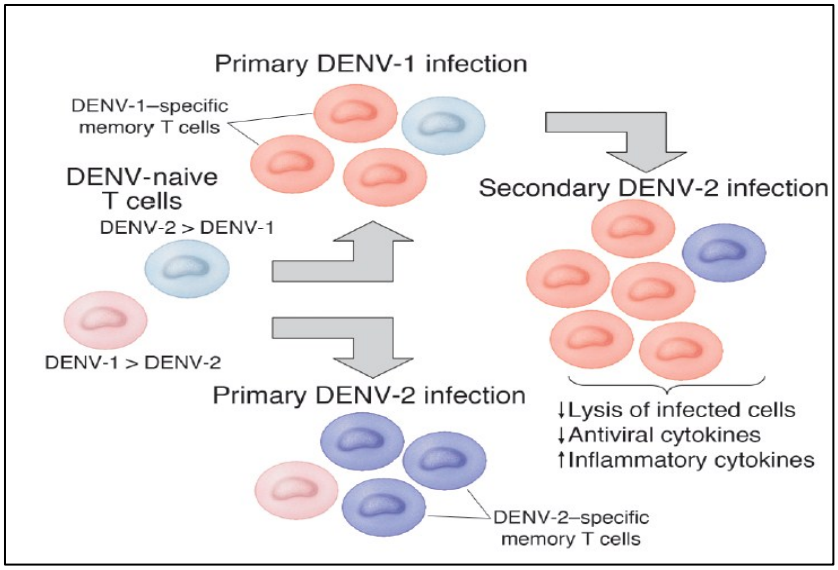


Figure 8. An example of a model of heterologous immunity in secondary DENV infections and its implications for the pathogenesis of DHF. In the Figure, DENV-1 was the serotype of the primary infection and DENV-2 was the serotype of the secondary infection for illustration purposes. The pale colors in the Figure represent the naïve T cell repertoire containing cells with higher avidity for DENV-1 than DENV-2 and other cells with the opposite avidity (red; DENV-1 to DENV-2, blue; DENV-2 to DENV-1). During a primary infection, T cells with higher avidity for the infecting serotype are expanded and enter the memory pool (represented by darker colors in the Figure). When DENV-2 infection follows DENV-1, the memory T cells with higher avidity for the earlier infection expand more rapidly than do naïve T cell populations. Due to these DENV-1 specific memory T cells having lower avidity for DENV-2, the mechanisms for viral clearance are suboptimal, while the proinflammatory responses contributes to the pathogenesis of the disease. Taken from Rothman, 2004. [104]

iv. Antibody-dependent enhancement (ADE) of infection. A person can be infected with DENV up to four different times, with each infection caused by a different serotype. A primary infection with DENV causes a robust, long lasting neutralizing response, providing lifelong immunity to reinfection with that same serotype, but subsequent infection by a different serotype may cause more severe disease.[21] Secondary DENV infection is the principal risk factor for severe disease in young children.[141] Primary infection of a person with passively obtained antibodies is also a major risk factor for DHF.[110] Infants infected with DENV during the second half of their first year of age can suffer from

severe forms of disease even though they are experiencing a primary DENV infection. This increased risk coincides with the waning of neutralizing activity of the maternal antibodies.

Partial immunity to heterologous infecting viruses can occur for up to 9 months after the primary infection, resulting in a milder secondary disease if the secondary infection occurred during this period.[38] Secondary infections occurring more than 9 months after the primary infection can cause more severe ADE disease.

ADE theory attributes the higher incidence of severe disease resulting from secondary infections to the following immunologic process: after an initial period of cross-reactive protection, antibodies from a primary infection remain cross-reactive to other DENV serotypes but at non-neutralizing levels. The non-neutralizing antibodies that bind to E or prM and to FcγR can enhance infection of the FcγR-bearing cells (such as infected monocytes). The specific types of FcγR that facilitate virus uptake and infection are FcγRIa and FcγRIIa. [142] Greater viral replication and immune activation would promote greater cytokine release which can damage endothelial cells. (Figure 7 and 8)

A differentiation has been described between extrinsic and intrinsic ADE. Extrinsic ADE results from non-neutralizing antibodies or neutralizing antibodies at concentrations below their neutralization capacity that enhance viral entry in the host cells by forming complexes with the virus and binding to FcR on the host cells. Intrinsic ADE is dependent upon the attachment and entry of virus-antibody complexes into FcR-bearing cells with the ability to modify innate and adaptive intracellular antiviral mechanism and thus enhance replication.[143] The host immune response to DEN disease (explained previously), has been shown to produce measurable differences in the metabolic profile of infected patients.[7]

I.C.6.b. Clinical outcomes. DENV clinical presentation is unpredictable; it can range from an asymptomatic, self-limited, mild disease that can go unnoticed to the most severe disease that can be fatal. Asymptomatic or subclinical disease is often the presentation of DEN. In these cases the individual

affected may not seek medical attention and the disease will be self-limiting. DF can be asymptomatic or symptomatic. DHF/DSS can occur during the second but not in subsequent heterotypic DENV infections. Only a small proportion of cases (5%) have a fatal outcome. With appropriate intervention, the fatality case rate in severe cases is less than 1%.The key sign that differentiates DHF from DF is increased vascular permeability that leads to a capillary leak syndrome, which can progress subtly and unexpectedly to DSS.

Appropriate prediction of DHF/DSS is critical for triaging patients and focusing hospital admission and treatment on those that will progress to the severe forms of disease.[100] This is especially important in DENV epidemics where public health services may be already overwhelmed by the sudden demand.

I.C.6.c. WHO 1997 guidelines for DEN diagnosis and case classification. These are the old guidelines used for DEN diagnosis and classification. This classification was developed based on pediatric cases in Southeast Asia and is still globally used.[144, 145] This system classified DEN cases based on disease symptoms into DF or DHF, the latter was further classified in four severity grades.[102]

The WHO 1997 guidelines defined DF by classifying it as undifferentiated fever or DF with hemorrhage or with unusual hemorrhage. DF is an acute febrile disease with two or more of these symptoms: headache, retro-orbital pain, myalgia/arthralgia, rash, hemorrhagic manifestations such as petechiae and positive tourniquet test or TT, (tourniquet test, to test capillary fragility, a cuff normally used to measure blood pressure is applied and inflated to a value in between the systolic and diastolic blood pressure for five minutes, the test is considered positive if there are more than 10 petechiae in one centimeter square) and leukopenia. [146]

DHF is defined as a disease that fulfills all of the following criteria: Fever or history of acute fever lasting 2-7 days and hemorrhagic tendencies evidenced by at least one of the following: a positive TT,

thrombocytopenia (100,000 platelets/µl or less), hemoconcentration evidenced by a rise of 20% or higher in the hematocrit value.[102]

DHF is classified in four grades of severity: Grade I is diagnosed by a positive TT. Grade II includes grade I and spontaneous bleeding in skin, nose or internal organs. Grade III includes grade II signs and hypotension for age, narrow pulse pressure of 20 mm Hg or less, restlessness and rapid weak pulse. Grade IV is determined by profound shock with undetectable blood pressure or pulse. DSS is constituted by grades III and IV. [102]

Some of the WHO 1997 diagnostic criteria for classification were found not to correlate with the disease outcome in several prospective and retrospective DEN studies. The TT was found to have poor sensitivity, ranging from 57% to 0%. The TT also was found to yield false positive results in some patients with non-DEN febrile illnesses, yielding a low specificity rate. The lack of diagnostic efficacy of the TT could be attributed in part to faulty blood pressure settings or the use of wrong sized cuffs. Further compromising the diagnostic efficacy of the TT, tests of patients with DSS who are poorly perfused could yield false negative results.[144]

Some of the diagnostic criteria proposed in the 1997 WHO guidelines were difficult to apply during DEN diagnosis. For example, a third of cases of pediatric DHF/DSS in Cuba did not have any obvious bleeding; a smaller proportion in Vietnam showed the same characteristics. These patients who did not have obvious signs of bleeding but presented with shock and evidence of plasma leakage were classified using the WHO 1997 classification as DSS in both countries.[147, 148] Occasionally thrombocytopenia may not be present in DHF cases who do not have low platelet counts; this occurred in up to 48% of cases in Sri Lanka and 78% in Cuba. [147, 149] It has been postulated that in mild DHF cases, the duration of thrombocytopenia could be brief and could be missed depending upon the timing of collection of the sample.

Meeting the WHO 1997 criteria for plasma leakage or increased vascular permeability can also be problematic. Capillary permeability/vascular leakage are most likely to appear 3 to 5 days after the onset of clinical signs; taking the samples too early would likely provide false negative results. In addition, to determine the presence of capillary permeability, the hematocrit should be elevated at least 20% above the baseline to account for escape of fluid, e.g. pleural effusion or ascites. However, hematocrit concentration could be easily masked by intravenous (IV) fluids administered to manage dehydration, severe anemia, blood transfusions or the inability to measure the hematocrit levels.

Because of these issues many cases of presumably DHF/DSS without hemorrhage and organ dysfunction would not fulfill the criteria of the 1997 WHO guidelines for DHF/DSS. [100, 144] New guidelines were developed by WHO to address these issues and to provide clinicians with a more useful system for diagnosis and patient management.

I.C.6.d. **WHO 2009 guidelines for DEN diagnosis and case classification; case definition guidelines**. Because of the problems when applying the WHO 1997 guidelines for DF and DHF/DSS diagnosis, new guidelines (WHO 2009) were developed that are based upon levels of disease severity. The diagnostic efficacy and disease classification utility were evaluated in a multicenter study conducted by the Tropical Disease Research branch of the WHO in 2006-7 which included patients from seven countries in Asia and Latin America. The new classification was released in 2009.[4, 6, 150]

In the WHO 2009 guidelines, a set of clinical and laboratory parameters differentiate between patients with severe and non-severe DEN.[6] The non-severe DEN group is subdivided in two subgroups: patients with warning signs (abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement, increasing hematocrit with decreasing platelets) and patients without warning signs (Figure 9). [6] The severe DEN group includes patients with severe plasma leakage, severe bleeding, or organ failure. [6] The criteria for the classification based on levels of severity are described in Figure 9.

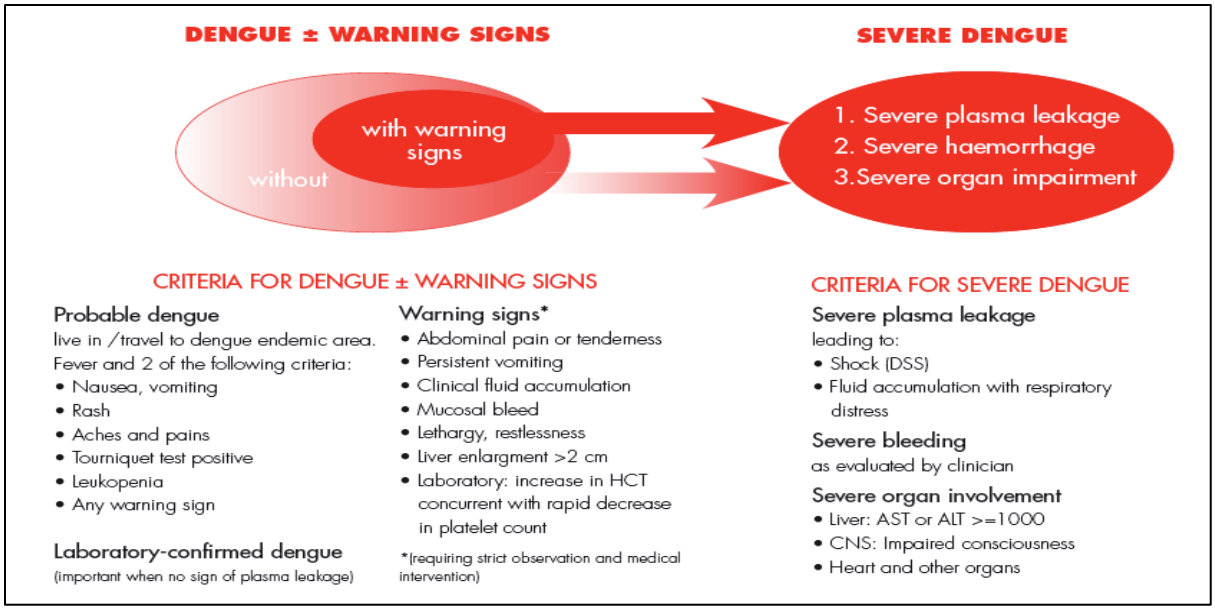


Figure 9. New WHO guidelines for diagnostic criteria for DEN case classification. Figure illustrates the criteria for the different classifications of DEN disease. Taken from: Dengue guidelines for diagnosis, treatment, prevention and control. WHO 2009.[6]

As mentioned, a number of hospital-based studies have been conducted to determine the relative diagnostic efficacy and clinical utility of the WHO 2009 guidelines as compared to the WHO 1997 diagnostic criteria and disease classification guidelines. A hospital-based pediatric study conducted in Nicaragua revealed that the revised WHO 2009 classification system is more sensitive in identifying severe cases that require specialized clinical attention.[4] A retrospective study in Taiwan revealed that approximately half the cases diagnosed as DF using the WHO 1997 classification had diagnostic criteria to be re-classified into the severe DEN groups of the new WHO classification system. These results suggest that the new classification system is more sensitive in detecting severe cases. Therefore it is a more useful approach to effectively diagnose and detect severe cases, which increases the opportunities for timely case management.[151]

The new classification system has been criticized by some researchers for associating diverse clinical responses into diagnostic categories that could obscure the underlying DEN pathophysiology, and thus may lead to inappropriate treatment and therapeutic intervention. There is also concern that

the WHO 2009 recommendation of hospitalizing patients classified as DEN with warning signs could result in over-admission of patients during epidemics, thereby reducing the efficiency and resources devoted to each admitted patient. [145, 152]

Nonetheless, the 2009 classification holds great value, and it is being currently evaluated in a multicenter clinical trial study to improve the guidelines postulated four years ago. The 2009 WHO classification was created to be used by clinicians and public health specialists dealing in the front line with the global pandemic of DEN disease, not to be a research tool in the understanding of pathogenesis. An improved case management by the opportune identification of potentially severe cases is more easily achievable with the 2009 classification. Being sensitive and simple, it enables the identification of a higher proportion of clinically severe cases, and it also allows the clinical spectrum of DEN to be captured by surveillance systems, which increases the comparability of epidemiological data.[153]

To implement a global change in the diagnostic classification of DEN is a difficult task. Even in this dissertation, as mentioned earlier, the old classification is used because in half of the samples (Mexican) the old classification is being used in the sampled locality. Nonetheless, an improved case classification, like the 2009 WHO DEN case classification, would be a valuable addition to the armamentarium of DEN diagnosis in all endemic localities.

I.C.6.e. Biomarkers of DEN. There is a need for biomarkers that can predict severity in DEN cases. Algorithms have incorporated clinical, virological and immunological results to identify early in the disease patients who will progress to severe forms of disease and who could be targeted for therapeutic intervention. To date these algorithms have not proven to be very accurate or useful. A number of studies have identified a variety of virological and host biomarkers associated with DEN disease severity. These include:

i. Virological biomarkers associated with hemorrhagic DEN disease include viremia titer, high concentrations of DENV RNA in the blood, NS1 antigenemia, [154, 155] heterologous DENV infections, [99], and infection with specific virus genotypes. [156] After isolating DENV from an infected serum sample, detectable differences in viral replication in cell culture could be biomarkers for virulence, including cytopathology, virus plaque size, virus replication efficiency, virus titer and/or the number of viral genome copies. It has been postulated that high titers of viremia, high titers of NS1 protein among others, could be predictive for severe disease.

ii. Clinical biomarkers associated with severe disease outcome include marked thrombocytopenia (platelet count < 100,000), hepatomegaly, liver tenderness and elevated liver transaminases aspartate aminotransferase and alanine aminotransferase (AST and ALT).[6]

AST is an important enzyme in amino acid metabolism, and is found in hepatocytes, heart, muscle tissue, kidneys, brain and red blood cells; it is a common biomarker for liver health. ALT also has a role in amino acid metabolism, having a key role in the alanine cycle. The liver is affected in severe cases of DEN, for this reason, finding liver enzymes elevated could be a potential biomarker for severe disease.

A proteomics study using two dimensional electrophoresis, mass spectrometry (MS) and Western blot analysis, determined that the proteins aldolase, alpha tubulin and thioredoxin peroxidase are all overexpressed in acute phase serum of DHF patients when compared to DF patients and thus could be potential prognostic biomarkers for severe disease.[157] Aldolase has been described to be elevated in skeletal muscle of mice inoculated with DENV and has been associated with myalgia in humans, a common symptom of DEN disease, it has a role in the metabolic pathway of both glycolysis and gluconeogenesis. Aldolase has three isotypes, (A, B, C), type A is expressed preferentially in the liver, type B in muscle and erythrocytes and C in the brain. It was postulated that aldolase (B) was elevated in the acute phase of the disease due to metabolic stress and in the convalescent stage due to metabolic

degeneration in the muscle as a result of cell injury causing myalgias. Thioredoxin peroxidase is commonly increased during oxidative stress due to immune activation due to viral infections. This protein could be associated with vascular permeability due to its role in the thioredoxin redox system, a regulator of metabolic cell functions and is also essential to maintain cellular homeostasis. Alpha-tubulin is a structural line of transport within the cell. It was postulated that this protein could have facilitated DENV entry into the cells. This study was conducted in a small population (9 DF and 9 DHF cases).[157]

Patients with DSS were found to have higher levels of nitric oxide than those with DF/DHF, suggesting that nitric oxide could be an important marker for DSS.[158] It has been demonstrated by gas chromatography-MS that NO regulates the contraction of smooth muscle and vascular tissue. NO can lead to vasodilation and reduction of systemic vascular resistance. This could cause hypotension, shock and death if not corrected. Clearly NO could be a relevant marker in the cases of severe hemorrhagic disease (DSS). Some oxidized lipid products like F2-isoprostanes, hydroxyeicosatetraenoic acid (HETEs) products, cholesterol oxidation products and arachidonate products have been found elevated in serum and urine of DEN patients.[159]

Clearly there are many potential biomarkers for DF and progression to DHF/DSS. However, there is not an accepted or standardized panel of biomarkers that could be used to predict the onset of severe disease.

I.C.6.f. DEN diagnosis. The attributes of an ideal DEN diagnostic test, beyond acceptable sensitivity and specificity requirements, include: 1) being useful in early diagnosis, able to distinguish between DEN and other DEN-like diseases; 2) being rapid, affordable, and convenient to use; and 3) using stable reagents at room temperature. Regarding epidemiological surveillance investigations, the test should: 1) be positive as soon as possible after onset of symptoms; 2) be specific, differentiate DENV serotypes, differentiate between primary and secondary infections; and 3) have a high throughput capacity.[160]

Detection of DENV analytes (i.e., virus, nucleic acid, or antigen) and demonstration of a DENV-specific human humoral immune response are the most widely used techniques for laboratory diagnosis of DENV infections. Current laboratory tests to detect DENV infections include: 1) detection of specific IgM or IgG antibodies or a 4-fold change in antibody titer in subsequent tests, 2) detection of DENV antigens such as NS1 or viral nucleic acids, principally by RT-PCR, 3) isolation of the virus, or a combination of the previous.

Virus can be isolated and viral antigens and nucleic acids can be detected in the serum (and other tissues) for 4 to 5 days after the onset of symptoms. The techniques are sensitive and specific but also tend to be expensive and require specialized laboratory equipment and personnel. After day 5, serological techniques are preferred for detection of immunoglobulins (IgM) for diagnosis because virus and viral analytes have been cleared from the blood. Serological tests are affordable and convenient, but the antibody response is typically too late to provide early diagnosis and prognosis of the disease in order to guide clinical management of the case if the patient should progress to severe forms of the disease (Figure 10).[6, 70]

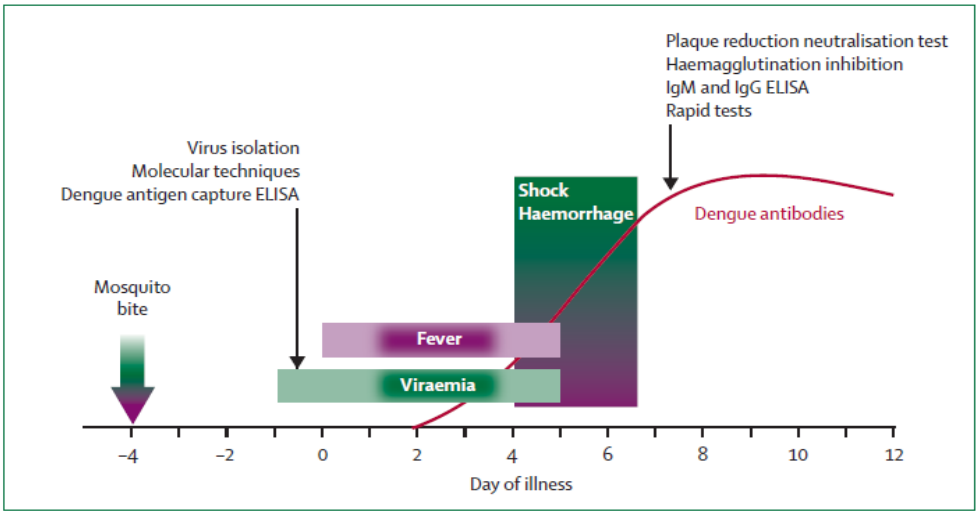


Figure 10. Timeline showing the disease course following DENV infection and the potential diagnostic tests during each phase of the infection. The diagram makes clear that serological tests would not be satisfactory to identify patients destined for severe DEN disease (DHF/DSS). Halstead, 2007 [70]

Brief descriptions of the most commonly used tests for diagnosis of DENV infection follow:

a. *Virus isolation* (VI) is the gold standard for diagnosis of DENV infections. This technique is useful for up to three days prior to the onset of fever, and up to 4 days after appearance of clinical signs and symptoms. After that time, virus has been cleared from the bloodstream. The most widely used VI technique is inoculation of serum samples onto *Ae. albopictus* C6/36 mosquito cell cultures or mammalian cell cultures such as Vero E6 or LLC-MK2 cells. Intracerebral inoculation of suckling mice can also be used to isolate DENV. Isolated viruses are then typically identified by immunofluorescent assays using serotype-specific monoclonal antibodies (mAbs). VI is very sensitive and specific, but this sensitivity is predicated upon properly collected and preserved samples and requires specialized laboratories and trained personnel, because infectious virus is used in the test. This technique can require several days, thus it is not recommended in cases when rapid diagnosis is required.[160, 161]

b. *Viral RNA detection* by reverse transcription-polymerase chain reaction (RT-PCR) amplification of DENV- RNA is a widely used technique that is rapid, sensitive and specific for DEN diagnosis. DENV-RNA analyte is detectable during the viremic period (up to 4 days after the onset of clinical signs and symptoms). Because of the exceptional sensitivity, this technique requires extreme caution to avoid false-positive results derived from contamination. It is also an expensive test and requires specialized equipment and trained personnel. [160, 161]

c. *Viral antigen detection:* Detection and quantification of NS1 has become a commonly used and convenient assay for DENV infection detection in acute phase serum samples. The sensitivity of the test may be lower in secondary infections compared to primary infections, due to the presence of cross reactive anti-NS1 antibodies that could bind to the antigen creating an antigen-antibody complex. A number of ELISA-based commercial tests for detection of NS1 antigen are available, but the tests can be relatively expensive. [160, 161]

d. *Immunohistochemistry:* This technique is not commonly used for diagnosis. It may be used to detect DENV antigen in *post mortem* examinations or in research laboratories to localize DENV antigens in cells and tissue sections using mAbs that can be labeled with enzymes or colloidal gold. [160, 162]

e. *Serological diagnosis.* Specific IgM and IgG responses are the most commonly used tests for diagnosis of DENV infections. ELISA-based kits are readily available for detection of DENV specific humoral immune responses. IgM levels increase starting on day 3 after onset of illness and reach the cut-off value for a positive diagnosis at days 5-10, and continue to increase for almost 2 weeks; this is most notable in primary infections. IgM antibodies persist for up to 6 months following primary infections. IgG antibodies to the primary infecting virus can be detected for life by ELISA.

f. *DENV neutralizing (NT) antibody detection:* Detection of DENV NT antibody is the gold standard for serological diagnosis. For diagnosis, NT antibody titers in acute and convalescent phase sera are determined. A $\geq$ 4-fold change in NT titer is serological confirmation of recent DENV infection. DENV serotype specific antibodies can be identified and measured by plaque reduction neutralizing tests (PRNTs) and other NTs. [161]

I.C.6.g. The potential for DEN diagnosis/prognosis using saliva and urine specimens. Currently, there is no accepted test to diagnose DENV infections using non-invasive clinical specimens. The standard diagnostic specimen is serum. Reports have been made of DENV analyte detection in urine and saliva. [163, 164] However, for DEN diagnosis there is no standardized non-invasive test. Serum is not an ideal sample to use for DEN diagnosis for numerous reasons, including resistance of a patient to subject him/herself to a blood draw, difficulty in bleeding children, and the potential for adverse events to occur, especially in children, when procuring the specimen. Further, obtaining the serum specimen requires sterile equipment and trained personnel, both of which are expensive. Non-invasive samples

such as urine and saliva are easier and less expensive to collect. [163] Interestingly DENV has been isolated from urine and saliva in one case report, in which the genome isolated from the samples was DENV-1.[165] NS1 has also been reportedly detected in urine in DF and DHF, with a low sensitivity (68% and 63% respectively).[166]

DENV detection in urine: In a case report concerning urine samples, the rate of DENV detection using RT-PCR was higher in samples collected on convalescent days as compared to acute phase samples. Urine specimens could be useful for convalescent diagnosis of DEN disease by RNA detection.[164, 167]

DENV detection in saliva: Saliva is an alternative non-invasive specimen for DEN diagnosis. DENV specific IgM can be detected in saliva. [163] Salivary IgM is a potential biomarker for DENV infection. IgA can be detected in saliva, but the sensitivity of the test was not satisfactory, and it is not recommended for DEN diagnosis.[163, 168] Oral swabs have been tested for detection of DENV- IgM, IgG and NS1 antigen; the detection of these analytes in swabs was less sensitive than detection of the same analytes in serum, but the specificity was satisfactory.[169]

I.C.7. Preventing and controlling DENV infections.

I.C.7. a. General therapeutic interventions. DEN treatment is limited by the lack of specific, effective and approved antiviral interventions. The typical management of a DEN case focuses on the treatment of symptoms such as fever, malaise and pain. Oral fluid administration with electrolytes and antipyretic treatment is recommended. Excessive vomiting or diarrhea and bleeding are indications for admission into a hospital. [6] These symptoms must be consistently managed with fluid replacement, preferably by oral administration when possible, and intravenous (IV) administration only if necessary. Sufficient liquids must be administered IV in order to replace fluid lost from the vascular system in order to avoid hypovolemic shock. If shock develops, administration of a bolus of 20mL/kg of IV solution is recommended until blood pressure is detectable. IV fluids, platelets or blood are reserved for patients as

a second-line therapy when they are not responsive or have developed severe bleeding or low platelet counts.[6, 102, 170]

I.C.7.b. Drug discovery efforts. Currently, there is not an approved clinical treatment for DEN disease. Different DENV proteins or steps in DENV replication are the focus of drug discovery efforts. The flavivirus replication cycle is being targeted for drug discovery and there is a large amount of information on potential targets in different parts of the replication cycle. Some of the potential targets include the NS3 protease, NS3 helicase, NS5 methyltransferase and NS5 RNA-dependent RNA polymerase.[171] A brief summary mentioning the logic of inhibiting some targets follows:

1) *NS3 Protease activity*: NS3 contains helicase, protease and RNA triphosphatase activities. The trypsin-like serine protease domain (NS3Pro), when associated with NS2B, plays a key role in the cleavage of the polyprotein, which is critical for viral replication. Preliminary attempts to block the enzymatic activity of this compound have been unsuccessful. Attempts to block the association with NS2 have potential as shown with studies done *in vitro* with HCV.[172, 173]

2) *NS3 helicase activity:* helicase is required for the separation of dsRNA formed during viral replication and the removal of proteins bound to the viral RNA during replication. Studies based on alterations of helicase, RTPase (RNA triphosphatase) or ATPase have shown the decrease or elimination of viral replication. [174]The principally targeted NS3 helicase location is the ATPase-binding site. NS3 NTPase and helicase has been shown to inhibit HCV, JEV and WNV helicases *in vitro*.[175] The bases for the interruption of this enzyme are not clearly understood.

3) *NS5 RNA-dependent RNA polymerase* (RdRp) is responsible for synthesis of the negative-strand RNA, using the genome as a template, making this an essential enzyme for viral replication. RdRp binds to the ds-positive-negative RNA duplex, along with NS3 helicase activity, and a new positive strand RNA is synthesized and capped by the combination of enzymatic activity. This enzyme is one of the most important targets for drug development because its

activity is essential for viral replication and human host cells are devoid of RdRp. Nucleoside analogs inhibitors can target the active site of the polymerase by binding to the RdRp domain; once bound, the elongation processes that the polymerase performs are terminated. This approach has been shown useful to inhibit BVDV *in vitro*.[176, 177]

4) *NS5 N-terminal methyltransferase:* The N terminal of the NS5 protein contains the methyltransferase (MTase) enzyme necessary for the formation of the mature RNA cap structure at the 5´ end of the viral genomic RNA. S-adenosyl methionine (AdoMet) functions as a methyl group donor for substrate modification; mutation of residues involved in NS5-AdoMet binding are capable of disrupting viral replication. [178] Unfortunately, the identification of compounds that inhibit specifically the binding of flavivirus MTase to AdoMet but not cellular activity have been problematic and so far been identified only by *in silico* screening.[171] Mutation of amino acids in the DENV MTase RNA cap-binding domain abolishes viral replication,[179] NS5 MTase proteins in all flavivirus have the same mechanism to bind GTP. For this reason the MTase RNA-cap-binding site is an ideal target to inhibit replication. [171]

5) *E protein*: Receptor binding is a crucial step for DENV entry in host cells; blocking the interaction between E and the host would provide an important avenue for therapeutic intervention that is currently being explored. Currently, the flavivirus receptor molecules have not been identified; therefore the blockage of the essential receptor that would directly inhibit viral entry is dependent on the identification of the specific receptor. E protein is the major target for the host humoral immune response and of neutralizing Abs against flaviviruses. Monoclonal antibodies (mAbs) directed against the lateral ridge region of DIII are very effective and could block virus entry, because that region is targeted by strong, serotype-specific neutralizing mAbs.

[171, 180, 181]

There is a large ongoing research effort to identify a potential antiviral for intervention or prevention of DEN severe disease. Currently, there is not a diagnostic test that can predict the development of severe disease or an antiviral that, given the prediction of a potential severe outcome, could treat it. A better understanding of DEN pathogenesis could help in the quest for both a diagnostics test and an antiviral.

I.C.8. Potential DEN vaccines. There is an urgent need for a DENV vaccine. Development of a safe and efficacious vaccine to prevent DENV infection and to induce a lifelong immunity against the four DENV serotypes has proven to be difficult. Currently, there is not a licensed DENV vaccine available to prevent DEN disease. The lack of a vaccine is in part due to the lack of an adequate animal model for DHF/DSS. Because of the possibility of ADE, it is critical to induce homotypic neutralizing antibodies against each of the four DENV, using a tetravalent vaccine. The vaccine should induce antibody titers to the E protein of each serotype. [38, 182] Several attempts are currently in progress to develop a safe and effective vaccine that can be made available in all endemic areas.

Some of the technologies used for vaccine development include live attenuated virus (LAV), purified inactivated virus (PIV), recombinant viral subunits, virus-like particles (VLPs), and plasmid or viral vectors. Some of these candidates are currently being evaluated in clinical trials and some other candidates are in preclinical development. Only live DEN vaccine candidates have been tested in large numbers of human volunteers. Several tetravalent DEN vaccines are being tested in clinical trials.

Some vaccine candidates that are or have been in various stages of human testing include 1) the live-attenuated chimeric yellow fever-DEN vaccine (phase 2b), [183] 2) a monovalent DNA vaccine consisting of a plasmid vector expressing DENV-1 prM/E (phase 1), [184] 3) a recombinant subunit tetravalent E-protein DENV vaccine (phase 1), [185] 4) a chimeric DENV-2 PDK-53-based tetravalent vaccine (phase 2).[186] A list of the vaccine categories available and examples of each will be summarized in the next paragraphs:

I.C.8.a. Live attenuated vaccines (LAVs). These vaccines theoretically produce strong, durable and broad immunity, including humoral and cellular immune responses, with one dose. Obtaining an acceptable level of attenuation with this type of vaccine can be difficult to achieve. LAVs are propagated in cell culture for their production. In this type of vaccine, DENV has been mutated typically by passage in cell culture or laboratory animals to infect but to replicate inefficiently in humans; the virus retains its ability to induce an immune response to structural and non-structural proteins. Concerns regarding genetic stability of LAV are based on the possibility of reversion to a more virulent phenotype during propagation in cell culture to prepare the vaccine or during infection and replication in the vaccinee.

There is a theoretical risk of recombination between vaccine and wild-type viruses. Finally, because of the potential for ADE, each of the four DENVs in the tetravalent vaccine must infect, replicate and induce a protective immune response in the vaccinee. Achieving a balanced immune response against the four serotypes of DENV in one vaccine has been very difficult. [38, 182] Frequently one of the viral serotypes is more fit for infection and replication, which results in a protective immune response to that virus, but an inadequate immune response to one or more of the other DENV serotypes, potentially conditioning the vaccinee for ADE and more severe disease during a secondary DENV infection.[38]

I.C.8.b. Chimeric virus vaccines based on the yellow fever vaccine. The ChimeriVax was created by substituting the prM and E genes from each of the four DENV serotypes for the same genes in the infectious clone of the YFV 17D vaccine. Phase I studies demonstrated that the vaccine is safe, and it elicits a low viremia titer and low infectivity for mosquitoes. However, phase 2b studies in Thailand showed no protection against DENV-2 infections, and an overall efficacy of 30.2%.[183, 187] The reasons for these disappointing results are being investigated.

I.C.8.c. Attenuated tetravalent DENV vaccines. Defined deletions were introduced in the 3' UTR region of DENV-1 and 4 cDNA clones. The mutations resulted in a balance between attenuation and

immunogenicity for DENV-1 and 4, but not for DENV-2 and 3. The DENV-1 and 4 vaccine candidates were safe and immunogenic; vaccine virus was not transmitted from vaccinees to mosquitoes. An alternative vaccine candidate was used to create candidates for DENV-2 and DENV-3. These vaccines candidates are broadly immunogenic and protective in rhesus monkeys. Phase 1 trials have shown this vaccine to be safe, but clinical evaluation has indicated that it has an unacceptably low level of infectivity in humans. Ongoing trials are testing newer formulations of this vaccine.[38, 188, 189]

I.C.8.d. Tetravalent chimeric live-attenuated vaccine. DENVax by Inviragen is based on an attenuated DENV-2 strain (16681-PDK53) as the backbone with prM-E genes from each of the four DENV subtypes. [190] In phase 1 testing, the chimeric viruses retained the safety properties of the original DENV-2 PDK53 vaccine and generated a strong immune response. The vaccine is currently undergoing a phase 2 trial.[191]

I.C.8.d. Nonliving DENV vaccines. Killed virus vaccines present reduced potential for inducing a long-lived immune response in contrast to LAVs (live attenuated vaccines), which theoretically induce lifelong responses following one dose of the vaccine. Multiple boosters of the killed virus vaccine are required to ensure lifelong immunity. To improve immunogenicity, adjuvants can be added although safety concerns have been placed in relation to adjuvants. Also adjuvants can increase the cost of a vaccine. [192] Killed virus vaccines theoretically can more readily produce a balanced immune response than LAV because of the lack of replication competition between tetravalent vaccine components. Thus they are able to induce a balanced response to E protein of each DENV serotype. Killed virus vaccines are considered to be safer than LAVs because there is no possibility of reversion to virulence. Some examples of this strategy follow:

I.C.8.e. Purified inactivated virus (PIV). This type of vaccine contains killed wild type virions and thus all DENV structural proteins, which permits induction of an immune response to prM, E and C

proteins. There is currently no candidate vaccine of this type in clinical trials. Preclinical trials are being conducted.[192]

I.C.8.f. Recombinant subunit DENV vaccines. In these vaccines, a portion of the E protein with deleted transmembrane domains or DENV E protein domain III (EDIII) are expressed, formulated, and used to immunize the host. This type of vaccine typically has low immunogenicity, making the addition of adjuvants a necessity. Some of the protein expression systems used to produce this kind of vaccine includes bacterial, yeast, insect and mammalian cells. Phase 1 clinical trials of some DENV vaccine candidates of this type are currently ongoing.[185]

I.C.8.g. Subunit vaccines-NS1 protein. Immunity to NS1 protein is associated with resistance in murine animal models challenged with DENV. One advantage of this type of vaccine is that immunity to NS1 will not elicit ADE, making it a much safer approach. Cross reactive antibody against DENV1 to 4 NS1 is detected after immunization with monovalent DENV-2 NS1. However the protection is serotype specific, and thus a NS1 vaccine would need to be multivalent. Its efficacy is still being determined.[38, 182]

I.C.8.h. Virus-like particles (VLPs). This type of vaccine offers significant potential. VLPs do not contain viral nucleic acids, C protein, or non-structural proteins. The VLP presents the E and prM protein antigens similar to what is found in the virion. The VLPs induce neutralizing antibodies and weak immunity to DENV in rodents. As with other killed virus vaccines, multiple doses are required to achieve immunity. DNA and virus vectored vaccines are frequently delivered to form VLPs. [193]

I.C.8.i. Virus-vectored DENV vaccines. Virus vectors can be used to express DENV genes to immunize hosts. For example, a replication-deficient adenovirus vector has been engineered to express tetravalent chimeric DENV E protein DIII. This type of vaccine is easy to deliver and has the capacity to express high levels of antigen in different cultured cells; in addition, these adenovirus-vectors are theoretically safe for humans. Pre-existing immunity to the adenovirus vaccine vector could decrease

effectiveness of this kind of vaccine; such antibodies could prevent uptake of the virus-vectored DENV vaccine into antigen-presenting cells (APCs.) To preclude issues with pre-existing antibodies, DENV antigens could be expressed by a Venezuelan equine encephalitis virus vaccine vector.[194] Measles virus vaccine vector (pediatric measles vaccine expressing a DENV tetravalent antigen) has also been developed and tested in mice, inducing strong neutralizing antibodies and cellular immune responses. It is in preclinical trial. [195, 196]

Despite intense efforts to develop an effective vaccine to prevent DENV infection, there is still not a vaccine available, and it is unlikely that a vaccine will be available in the near future. For this reason it is important to focus and improve the efforts in surveillance and vector control to reduce DEN cases.

I.D. MOSQUITO-BASED DENV SURVEILLANCE.

DENV surveillance is critical to prevent DEN outbreaks and epidemics. Currently, passive surveillance for DEN cases is used in most countries. However, laboratory identification and reporting of cases may not be consistent and reporting of cases is typically not available in a time frame to permit intervention in impending outbreaks, and case reporting is frequently low during inter-epidemic periods.

In addition, most DENV infections are asymptomatic, which complicates further the use of passive case reporting as a surveillance mechanism for impending epidemics. Clearly, passive case reporting is not an effective surveillance approach if the aim is to implement prevention and control measures in an opportune manner before epidemic transmission begins. [197]

Mosquito-based surveillance could clearly be of value for DENV surveillance and for targeting areas at greatest risk for control measures. Because mosquitoes are not as mobile as human beings, detection of virus in mosquitoes provides much greater spatial and temporal localization of where transmission is occurring. In addition, even in the case of asymptomatic infections in humans, DENV will still be detected in mosquitoes, providing invaluable early information for public health authorities on

which DENV serotypes are circulating, or whether a new serotype or genotype have been introduced, etc.[69]

Active or proactive surveillance for human cases provides more timely information on impending epidemics. Public health agencies can target clinics in areas for enhanced surveillance and reporting of DEN cases. In some circumstances, early warning of DENV activity could be obtained by monitoring the incidence of presumptive (clinically diagnosed) DEN cases instead of laboratory confirmed cases. This would allow public health practitioners to implement interventions earlier (theoretically several weeks earlier) than waiting for laboratory confirmation of DENV infections. For this kind of proactive surveillance to properly function, it is vital to have a functioning sentinel clinic and physician network, an alert system based on fever cases reported by community health workers and a sentinel hospital system. [69] Even then, the predominance of asymptomatic DENV infections makes case reporting a less than ideal approach for DENV surveillance and implementation of control measures. Proactive surveillance that includes both epidemiological and entomological components could provide better predictive capability for impending DENV epidemics. Combining information on vector abundance and disease risk could be used to target source reduction and insecticide spraying campaigns to areas at most risk for DENV epidemics. This strategy includes monitoring for the presence and abundance of *Ae. aegypti* using larval, pupal, and adult indices.[198] However, currently used entomological indices have little value in predicting DENV epidemics; they are useful principally for targeting control measures, for example reducing or eliminating water-holding containers in the home environment. [69, 198]

Weather factors, including monthly average temperature and rainfall, and the indoor abundance of *Ae. aegypti* females have been positively correlated with the incidence of DEN cases in the following months. Nonetheless, the significance of these factors, although highly relevant for

surveillance, is limited for DEN case prediction, due to the serotype-specific susceptibility of the human population to DENV that influences the incidence of DEN cases.[199]

CDC recommendations are usually followed for mosquito-based surveillance. Adult female mosquitoes are collected in oviposition traps, sticky traps, light traps or CO2-baited traps, and pools of adult mosquitoes are tested for the respective virus or its nucleic acids or antigens using virus isolation, RT-PCR, or ELISA, respectively. RT-PCR is the preferred approach; however it is expensive, requires trained personal and appropriate equipment. [200]

For effective surveillance, mosquito traps must be collected timely to ensure viability of the mosquitoes and the viruses in them. Occasionally in remote locations, traps cannot be readily collected, leaving them for periods of time longer that what conventionally is established for their collection. This causes the mosquitoes to dry, potentially making surveillance ineffective.[201, 202] In addition, processing of mosquitoes typically requires multiple freeze thaw cycles, which can inactivate viruses and destroy analytes. A test that avoids these pitfalls for mosquito-based surveillance would be of great value and could be fruitfully applied for DENV surveillance. Two major advances have made mosquito-based surveillance for DENV more realistic 1) dramatic improvements in traps for *Ae. aegypti* and the use of backpack aspirators now permit collection of large numbers of *Ae. aegypti* females for analysis [203], 2) and the feasibility of detection of NS1 antigen in mosquitoes using commercially available kits. [204, 205]

Incorporation of mosquito-based surveillance could increase the effectiveness and sustainability of DEN control programs. *Ae. aegypti* mosquito control has proven difficult and elusive to achieve and to maintain by public health authorities. There are not effective DENV surveillance and mosquito control programs in most endemic countries.[197] Vector control programs are typically under-resourced and overwhelmed trying to control *Ae. aegypti*, which is now essentially hyper-abundant throughout the tropical world.

I.E. METABOLOMICS AS A STATE-OF-THE-ART PLATFORM FOR THE DETECTION OF SMBS OF DENV INFECTION IN BIOLOGICAL FLUIDS AND PREDICTION OF DISEASE OUTCOMES.

I.E.1. Metabolomics and its role in the "omics" cascade. Monitoring of metabolic changes in humans to detect diseases is not new; physicians have for millennia looked for changes in the color of urine and saliva of their patients to diagnose an illness.[206] Exciting advances in instrumentation and analytical software now provide unprecedented opportunity to identify metabolic changes. The metabolic processes of organisms are perturbed by changes induced by, for example, virus, bacterial or parasitic infections. Metabolites that change quantitatively or qualitatively can be identified in diseased and non-diseased states and directly attributed to infection by relating host responses to the pathogen and tissue damage, inflammation and other microbial induced pathology to host metabolic pathways.

Metabolites or SMBs are the chemical entities that are transformed in metabolism and provide a direct functional panorama of cellular biochemistry, state and activity that can be correlated to the phenotype. In metabolomics, SMBs (physiologically relevant metabolites) are discovered, structurally characterized, and quantitated. This information provides insight into the basic biochemistry and pathways involved in metabolite generation and increase our understanding of comprehensive cellular metabolism and important biomedical states. [206-210]

Metabolomics is an exciting new discipline that focuses upon the analysis of low molecular weight molecules that result from cellular processes; it addresses the entire set of small molecules (<1500 Da) in a biological sample. These small molecules have very important roles in disease etiology and treatment. [211, 212]. Unlike proteins, transcripts and genes, the metabolites are not encoded in the genome. This makes the metabolites less prone to cataloguing than the objects of study from the other "omic's" disciplines. Genes and proteins are subject respectively to regulation by epigenetic processes and post-translational modifications, while metabolites are representative of the downstream biochemical end products of the phenotype.[207]

Mass spectrometry coupled with sophisticated statistical analysis to associate metabolites with disease conditions and bioinformatics to identify the metabolites of infectious diseases outcomes provide innovative opportunities for identifying small molecule biomarkers (SMBs) and pathogenic mechanisms and pathways. Such information can be exploited for development of novel tests for disease diagnosis and prognosis.[213]

Metabolomics, when coupled with proteomics and genomics, can help gain insight in the pathological or physiological processes of the cell, gene expression, protein expression and enzyme activity by measuring biochemical function directly by monitoring the substrates and products transformed during cellular metabolism.[206]

The metabolic activity of host cells and tissues is influenced strongly by disease and environment, which can perturb metabolism and the physiologic state of cells, resulting in a change in the profile of metabolites. For infectious diseases, the metabolomics profile will likely differ between individuals infected with a pathogen and those not infected with the pathogen. Metabolomic profiles may also differ between individuals infected with the same pathogen but who are experiencing or will experience different disease outcomes (e.g., DF vs. DHF/DSS). Currently, acute infectious diseases diagnosis is predominantly based on pathogen-detection and/or the detection of the host's immune response to the pathogen. Metabolomics can provide important tools for the pursuit of a SMB diagnostic panel.

I.E.2. Untargeted and targeted metabolomics: Two basic approaches used for the metabolomics studies presented in this dissertation are: 1) untargeted or fingerprint studies and 2) targeted or footprint studies.

I.E.2.a. Untargeted metabolomics. Metabolomic fingerprinting is an unbiased approach that describes and compares the pattern or fingerprints of all metabolites included in the samples, especially in the ones that are affected by pathological states like exposure to toxins, diseases, and genetic or

environmental alterations. The type and characteristics of metabolites detected will depend on the sample preparation and MS analysis. Metabolomic fingerprinting or untargeted metabolomics has revealed that the number of endogenous metabolites in biological systems is larger than expected and is not completely accounted for by merely canonical biochemical pathways. The number of annotated metabolites in databases has increased dramatically over the past decade, facilitating untargeted metabolomics studies; however, the chemical structure, cellular function, anatomical location and metabolic pathway of many metabolites remain uncharacterized. [207, 213]

I.E.2.b. Targeted metabolomics. Metabolomic footprinting is a hypothesis-driven strategy for analyzing the properties of cells or tissues with a high-throughput approach that is based on specific metabolic pathways or a class of compounds. Metabolites are selected prior to analysis and specific analytical methods are then designed for the metabolites' identification. [213, 214]

I.E.3. Metabolomics in biological fluids. Defining the metabolic profile of biological fluids, including serum, urine, saliva and cerebrospinal fluid, is the subject of much research. Defining the metabolome of these specimens in healthy individuals is critical to detect differences in the respective metabolome of diseased individuals. A brief description of what is known regarding the metabolome of the respective biological fluids follows:

I.E.3.a. Serum Metabolome: Serum is a constituent of the blood, which circulates through every tissue and organ. It essentially is a liquid circuit for all the molecules that are being secreted, excreted or discarded by different tissues in response to physiological stimuli or stress. Serum contains a large number of metabolites that vary under physiological conditions or under pathological states. [215] Serum lacks fibrinogen, prothrombin and other clotting proteins. It contains proteins and peptides, (e.g. albumin, globulin, lipoproteins, enzymes), hormones, nutrients (e.g. carbohydrates, lipids, amino acids), electrolytes, dissolved gases, organic waste, and other molecules of the metabolome.

The most common ions found in serum are sodium, chlorine, bicarbonate/carbon dioxide, iron, oxygen, potassium, calcium, phosphorus/phosphate and sulfur/sulfide and magnesium. These ions play central roles in the maintenance of homeostasis in cells and membranes, and this translates to healthy organs and tissues. Disturbances in any of these ions could lead to a loss of metabolic homeostasis in the cells. Twelve of the most abundant organic metabolites in serum are D-glucose, total cholesterol, melanin, urea, ATP, glyceraldehyde, cholesterol esters, L-lactic acid and fructosamine, L-glutamine, L-alanine, methanol, glycine, L-lysine, uric acid, and hydroxybutyric acid. The least abundant metabolites include diacylglycerols, vitamin K1, 13,14-dihydro prostaglandin E1, substance P (a neuropeptide), and prostaglandin E1, various glycerophospholipids, vasopressin, 11-trans-Leukotriene C4, nitric oxide, thyroxine, 3,5-diiodothyronine, epietiocholanolone, thromboxane B3, thyroxine sulfate and 11b-hydroxyprogesterone. The current lower limit of detection of these compounds in serum is in the low picomolar range, and the concentration range of compounds spans close to 11 orders of magnitude.[212]

The different classes of compounds found in “normal-healthy” serum are currently being determined; by knowing the concentration of these biological compounds in healthy serum, we will be able to detect anomalies in the metabolism caused by disease.

I.E.3.b. Urine Metabolome. Urine is affected by homeostasis regulation; it is a waste product and possesses significant information regarding metabolic disregulation which can provide insights into the system’s metabolic changes that can reflect physiological or disease processes.[216, 217] Urine from a healthy person is composed of approximatedly 95% water; the rest is composed of organic molecules such as urea or carbamide derived from ammonia produced by the degradation of amino acids. Creatinine, an important constituent, is produced from creatine phosphate in the muscle tissue. Uric acid and small amounts of carbohydrates, enzymes, fatty acids, hormones, pigments, and mucins can also be found in urine. Ions that preserve a delicate equilibrium with kidney filtration include among

others sodium, potassium, chloride, magnesium, calcium, ammonium, sulphates and phosphates. The changes in the concentration of compounds resulting from pathogen infection could be used in a biomarker panel for detection of disease.

Urine also presents analytical challenges for metabolomics analysis. Variations in ionic strength, variations in pH, osmolarity, nocturnal and diurnal variations and other variations induced due to physiological stress can complicate metabolomics analyses. Urine also varies in its dilution, and thus in its range of metabolite concentrations. It also contains microbial metabolites and it is also affected by drugs, diet, pollutants, and industrial chemicals that may be cleared from the system by the urine. [217, 218]

I.E.3.c. Saliva Metabolome. Saliva offers a great opportunity for the development of serum-alternative molecular diagnostics because it contains most components found in serum. Saliva's potential has been largely underestimated principally due to the limitations on the available technologies necessary for the screening of the complex constituents that may be found at low abundance in saliva. New promising technologies with greater sensitivity are now available to screen and identify saliva components successfully. [215, 219-221]

Saliva can reflect the physiological state and variations in the body, which include nutritional, endocrinal and emotional induced metabolic changes. Saliva contains enzymes, hormones, antibodies, antimicrobials, proteins, nucleic acids and cytokines which enter saliva from the blood that increase the complexity of metabolomics analyses. Proteomics studies have revealed salivary secretory proteome components, their post-translational modifications and protein complexes and their functions. The salivary proteome could serve to determine the status of oral and general health.[220]

Salivaomics is a term coined in 2008, and reflects the study of the transcriptome, metabolome, proteome, microRNA and microbiome of saliva. Defining and understanding the saliva metabolome is still a work in progress. [220]

Many metabolites remain to be identified in serum, urine, saliva and other biofluids; the number of identified compounds in the major metabolomics databases (Human Metabolome Database (HMDB), Metlin, InChI (International Chemical Identifier), Lipid Maps, ChEBI, Pubchem, Mass-Bank, Serum metabolome database (SMDB) and ChemSpider) increases almost daily, adding new compounds to open databases. More research is needed in order to achieve a comprehensive database of the metabolome present biofluids.

I.E.4. External influences in the metabolome: Many external factors can perturb the metabolome and change the expected results. Factors such as age, diet, body fat, sex, and other environmental factors are potential causes of variance in the metabolome. These differences can be taken in account when the sample size is large enough. Brief description of some of the most relevant factors follows:

I.E.4.a. Aging. Changes in cell organelles, organs and systems occur during the process of aging. This leads to changes in physiology and in the metabolism that affects the whole body homeostasis. Aging is characterized by progressive decline in cellular function. Aging effects can be caused by the accumulation of free radicals and glycated proteins, chromosome telomere shortening, hormonal dysfunction, chronic-low grade inflammation, and other changes.[222, 223] For example, lifestyle and surroundings could also play a role in the metabolic changes with age. [222]

There is a positive correlation between oxidative stress and the metabolism of sphingomyelin, which is an important component of cell membranes. Sphingomyelin has a role in membrane fluidity and favors signal transduction. Aging causes a decrease in the long chain acyl carnitines and increase the free fatty acid levels. Studies have shown that the altered sphingomyelin metabolism that increases its degradation to ceramide is accelerated by oxidative stress. [223, 224] Recent evidence suggests that all aging pathways known so far including DNA damage, accumulation of reactive oxygen species and general metabolic dysfunction are interconnected.[222, 223]

I.E.4.b. Sexual dimorphisms. Gender-related differences in the serum metabolites have been described; these differences are also likely present in saliva and urine. Skeletal muscle mass varies between females and males of the same age, and it is an important determinant of energy requirements including fat oxidation and insulin sensitivity. [225]

I.E.4.c. Diet and Weight. Reduced or increased body mass can also perturb the metabolome. Changes in the concentration of amino acids, acylcarnitines, phosphatidylcholines and sphingomyelins are some chemical classes of compounds that can vary in serum due to variations in body weight. Obesity during childhood can cause changes in the metabolome. Comparison of the metabolome of obese and normal-weight children revealed that glutamine, methionine, proline, and phospholipids differed the most between the two groups. These metabolites are commonly indicative of oxidative stress and modifications of sphingomyelin metabolism. Interestingly, pubertal stage was not associated with metabolite concentration difference.[226, 227]

I.E.5. Metabolomics Analysis Platforms for Biomarker Identification.

I.E.5.a. **Introduction.** Currently there is no single metabolomics platform or instrument that can analyze all the metabolites in a sample. Protocols for extraction, sample preparation and analytic techniques that are useful for one class of metabolites could be completely useless for other classes of metabolites.[210, 213, 228]. The basic sample processing and analysis flow for a metabolomics study is provided in Figure 11 In this study, the steps in the right column circled in red were followed to tentatively identify SMBs by LC-MS/MS. A brief review of the workflow steps, including mass spectrometry approaches, data analyses and metabolite identification follows: Basically, the experimental approach consisted of collecting samples, preparation of samples, sample analysis by liquid chromatography and mass spectrometry, metabolite detection, data export and analysis, and finally metabolite identification (Figure 11).[229]

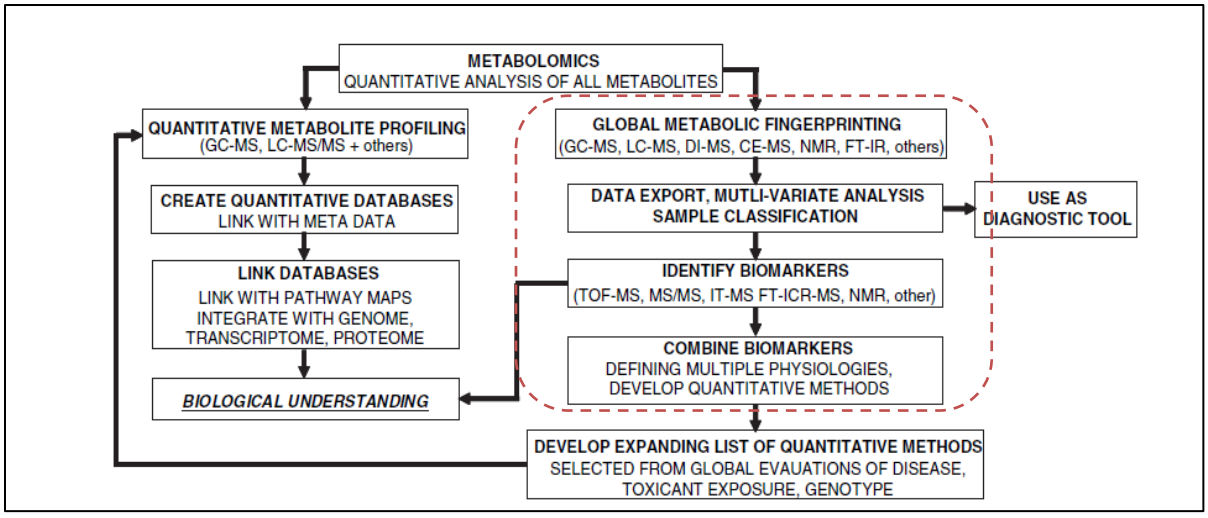


Figure 11. Metabolomics workflow. Experimental workflow of metabolomics analysis illustrating the steps and strategies that can be followed for investigations. Circled in red are the steps followed in this investigation. Taken from Dettmer, et al 2007.[213]

I.E.5.b. Analytical Sample preparation for metabolic fingerprinting. Metabolic fingerprinting can be conducted using liquid or solid tissue samples. In the current study serum, urine and saliva specimens were analyzed. Regardless of the sample selected, it must be processed appropriately before analysis. Biological samples are very complex; a preliminary separation is commonly done before mass spectrometry, which will promote superior identification of metabolites. Sample preparation is a very important, challenging, and error-prone process of the analysis. It consists of simplifying a complex biological matrix, such as serum, saliva or urine, into a format that is compatible with the analytical technique used. Several approaches for protein precipitation have been developed; their main purpose is to remove, using organic solvents, matrix components such as peptides or proteins (more abundant in serum) that could potentially interfere in the mass spectrometry analysis. The most commonly used solvents are methanol, acetone, acetonitrile and chloroform. During this process, there will be loss of metabolites due to enzymatic activity or oxidation processes, which could impact the study and understanding of the metabolome. Freezing the sample soon after collection could help reduce the impact of sample degradation. [207, 213, 230]

Serum sample processing. Serum is a biofluid with extreme molecular complexity. Preparation of serum helps increase reproducibility; exposure to contaminants needs to be minimized during preparation. A standard preparation is focused on eliminating the excess of proteins in serum to simplify the sample introduced into the mass spectrometer and to avoid saturating the detector with large molecules. Details about serum preparation for this project can be found in Chapter 4.

Urine sample processing. The preparation of urine samples for mass spectrometry analysis is simpler than serum preparation. Urine is a less complex specimen due to lower protein content in healthy individuals and to smaller size and higher thermodynamic stability of urinary peptides, which will reduce intermolecular interactions; therefore molecules are less complex.[216, 217]

A typical protocol for preparation of urine for analysis by mass spectrometry consists simply of specimen centrifugation followed by dilution with water; this simple protocol theoretically minimizes loss of analytes. [217] More details about urine preparation protocol for this project can be found in Chapter 4.

Saliva sample processing. Saliva preparation for mass spectrometry analysis has been the subject of many studies. [215, 221, 231] The approach has been to optimize the process by reducing the number of proteins present to improve the reproducibility of saliva analyses. More details about saliva preparation for this project can be found in Chapter 4.

I.E.5.c. **Sample analysis-Liquid-Chromatography.** Before analyzing the sample in a mass spectrometer, the prepared specimen is typically processed by a chromatography-related technique in order to optimize the separation of its molecular components. Gas chromatography and capillary electrophoresis are some of the available technologies for chromatography separations, but this review will focus on liquid chromatography.

Liquid chromatography (LC)is frequently interfaced with the mass spectrometer (MS) instrument. LC, being done previously to MS, helps reduce ion suppression that coeluting compounds

can produce by reducing the number of competing compounds by separating those molecules before they reach the MS. It also can separate isomers, improving the detection limits and MS data quality by decreasing background noise, all of which favor repeatability. [217] LC can be performed in normal or reverse phase, and sensitivities in the pg/mL range can be achieved. [228] In-depth details about liquid chromatography protocols will be presented in Chapter 4.

I.E.5.d. LC Analytical Columns: normal and reverse phase. Chromatographic separations require an analytical column. A variety of columns and techniques are available for LC, including: partition, normal-phase, reverse-phase, hydrophilic interaction chromatography (HILIC), size-exclusion, ion-exchange, bioaffinity and aqueous normal-phase chromatography. The columns differ in their tolerance for pump pressure, pore sizes, and the polarity of compounds retained and eluted, among other attributes. Each column for hydrophilic interaction liquid chromatography (HILIC) and reverse-phase chromatography (RP) provides different significant biomarkers that, combined, enable coverage of most of the metabolites present in the biological samples of interest. [232, 233] Normal-phase or HILIC chromatography and RP chromatography were selected for the analyses presented. HILIC separates analytes based on their affinity for a polar stationary surface such as silica; reverse-phase has a non-polar stationary phase and a moderately polar mobile phase. The vast majority of the analyses in this dissertation were performed with HILIC (Cogent Diamond Hydride); for this reason it is described in detail.

i) Hydrophilic interaction chromatography (HILIC) Analysis: HILIC can be used for metabolic targeted and non- targeted studies in a high performance liquid chromatography (HPLC) system coupled to a quadrupole time of flight (Q-TOF) mass spectrometer. It works well with electrospray ionization (ESI) used to ionize the molecular features. HILIC consistently yields better retention of the polar/ionic analytes that elute very early or even are not retained in conventional RP analyses. [232, 233]

HILIC is a variant of normal phase LC. It separates molecules based on hydrophilic interactions of the stationary and mobile phase with polar metabolites that provide strong retention and higher compatibility and sensitivity in LC-MS systems. Highly polar metabolites can include simple saccharides, most amino acids, aminated bases, nucleotides, short chain organic acids, phosphate molecules, among others. These types of molecules play a very important role in many vital biochemical processes, but because of their polarity, they are not well retained by standard RP-LC columns.[234-236]

The HILIC separation mechanism is not completely understood. [233, 236] The analyte retention mechanisms that HILIC utilizes for separation are partition, ion exchange and dipole-dipole interactions of the eluent with a water-enriched layer that covers the stationary phase particles. HILIC occurs when a pseudo-stationary phase is created with the support of a bonded phase, and an aqueous layer is adsorbed in its surface.[233] (Figure 12)

The major drawbacks of HILIC include an intensive consumption of expensive acetonitrile (ACN), long equilibration times; peak shape distortions, retention time shifts and lack of solubility of some compounds in high proportions of organic solvents.[234, 236]

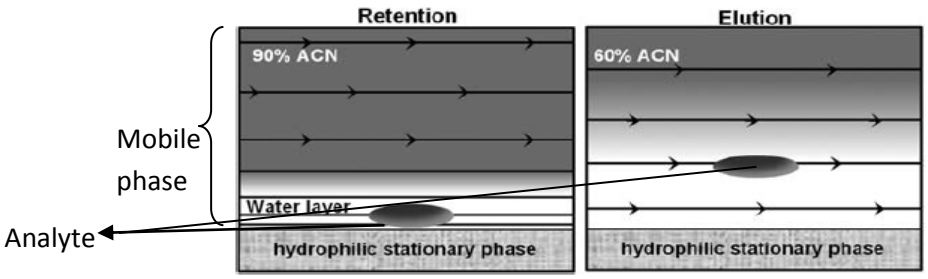


Figure 12. Scheme of HILIC separation of a polar analyte in the water layer on the surface of the hydrophilic phase. At higher concentrations of ACN (>90%) polar analytes are partitioned into the water layer adsorbed (retained) on the surface of the hydrophilic phase. Non-polar analytes will prefer association with ACN and move through the column with little retention. Polar analytes will associate with the stationary phase and be retained. The water layer adsorbed onto the stationary phase competes with the nonpolar mobile phase to allow liquid-liquid partitioning of analytes. As the concentration of ACN decreases, the polar analyte elutes from the column.(from Greco, et al.2013) [233]

HILIC Stationary Phase: A stationary phase is the part of the column through which the mobile phase flows and where the distribution of the compounds between phases occurs. When the goal is to retain and separate polar compounds, the stationary phase should be polar. The phases are bonded to silica supports, which are not inert under HILIC conditions. Silica gel is one of the most common stationary phases. Silica hydride (type C silica) (Figure 13) has been recently introduced in metabolomics; it has less than 5% Si-OH and may carry approximately 2% bonded carbon on the surface. Therefore type C silica is less polar than ordinary silica (Figure 13). This is the column used in the experiments presented in Chapters 2 and 3. This approach is also called ANP or aqueous-normal phase. Type C silica column offers improvements in retention reproducibility and faster equilibration than other normal-phase columns.[234]

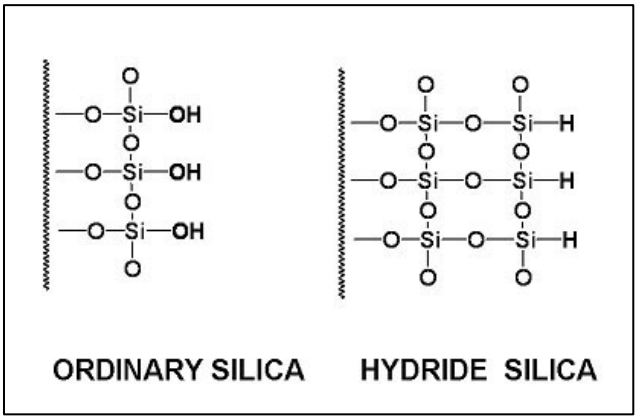


Figure 13. Modified HILIC column used for separation in current experiments. On regular HILIC columns, polar compounds partition into and out of the hydration shell created by adsorbed water on the silica surface. As the acetonitrile concentration increases the water layer decreases (Figure 12) and the charged polar analytes are retained by the combination of cation exchange with the silanols under the water layer and the partitioning effect. The combination of these two mechanisms retains polar compounds in HILIC mode. On the hydride silica (the one used for this project) the charged polar compounds elute in a similar order as on HILIC columns; however non-polar compounds will be retained at the same time by the non-polar phase of the columns which has a small hydrocarbon bonded phase on a silica hydride surface.

HILIC Mobile phase: The mobile phase should contain at least a minimal amount of water and be mainly organic, with a lower content of hydroxyl groups (e.g. 90% acetonitrile, 10% water) to form a water layer on the stationary phase surface for polar analytes to be partitioned and retained. Protic

solvents (water) act as a strong solvent; thus HILIC requires water-miscible polar organic solvents like ACN or methanol.[213, 228] Elevated levels of organic content present in the mobile phase will cause polar compounds to be highly retained in the water-rich liquid layer (Figure 12). If an elevated salt concentration is present, it could cause more solvated salt ions into the water-rich liquid layer. These ions could be competing with the analytes of interest. [233]

ii) Reverse phase (RP) chromatography. RP is successfully used for the separation of hydrophobic metabolites. RP is preferred for the separation of medium and non-polar analytes; very polar analytes are not retained and will elute in the void volume. One alternative for RP methods is UPLC (ultrahigh performance liquid chromatography), which employs RP columns with a smaller particle size than traditional and can improve the sample separations and upgrade the resolution.[207, 228]

The datasets derived from RP-LC-MS analysis are less robust, but much more sensitive and informative than the ones generated using normal phase. The metabolites detected will cover a broad range in molecular weights, polarities and physicochemical properties; the analytical conditions are easily modified for profiling or fingerprinting. There is also a high reliability and exchangeability between different instrumentation manufacturers, robustness of the analytical conditions and good reproducibility from batch to batch.[234]

I.E.5.e. **Ionization.** There are several options available for ionizing compounds in a metabolomics analysis. This can be divided in soft ionization that includes matrix assisted laser desorption ionization (MALDI), ESI, atmospheric pressure chemical ionization (APCI), and fast-atom bombardment (FAB). APCI induces limited fragmentation, which can be useful for the analysis of non-polar and thermally stable molecules, including lipids, and it can be used coupled with ESI to improve the analyte ionization and hard ionization. Hard ionization techniques require vaporization of the analyte from which ions are generated from the neutral molecules that have been introduced into the gas phase. This process causes

fragmentation of the molecule. The preferred biomolecules for hard ionization are small, volatile molecules. Common modes of hard ionization include electron impact (EI) and chemical ionization (CI).

For the analyses presented ESI was selected. ESI is the most common type of ionization coupled to LC-MS. ESI offers a soft ionization, which is preferred for polar and non-volatile biomolecules such as peptides, proteins, lipids, oligonucleotides, polymers, nucleic acids, non-covalent complexes and oligosaccharides. ESI keeps the molecule intact, allowing observation of the charged (M+) ion.[237]

I.E.5.f. Mass spectrometers. The quadrupole (Q) and time of flight (TOF) are the most commonly used mass spectrometers. Ion traps, Fourier transform, and orbitraps are some other analyzers available for metabolomics. Quadrupole (magnetic source that attracts or rejects a charged ion) are easily used with many different ionization sources, although they are typically coupled to ESI. A quadrupole instrument needs three quadrupoles placed in series to perform tandem mass analysis. The function of the first quadrupole is to scan a preset *m/z* range to select an ion of interest, which is fragmented in the second quadrupole; the collision cell will use either argon or helium as the collision gas to fragment the molecule. The third quadrupole function is to analyze the ions fragmented in the collision cell. In ideal calibrating conditions, TOF instruments have high resolution, fast scanning capabilities for mass spectrometry and accuracy of up to 5 parts per million (ppm). The quadrupole mass analyzer can be used for both MS scanning and MS/MS studies.[228]

I.E.5.g. Data-handling. Statistical tools for metabolomics data analysis need to be selected according to the purpose of the study in order to optimize the results obtained from the LC-MS. Once the LC-MS or LC-MS/MS data are collected, the data files produced from the specimen analysis need to be carefully analyzed. Specialized software (MassHunter, Mass Profiler Professional, XCMS, among others) makes it possible to analyze complex datasets produced by LC/MS. [207]

Features (compounds with a mass and a retention time) are extracted from the data file collected from every sample. Hundreds to thousands of features can be found in biological samples

such as serum, saliva and urine. A molecular feature extraction (MFE) algorithm is used for this purpose. MFE identifies peaks in the total ion chromatogram raw data, reducing the complex data set to a simple list of potential chemical formulas/compounds by use of accurate mass, retention time, intensity and a list of ions potentially associated with each peak. The files can then be grouped by diagnosis groups. The features obtained from MFE will later be filtered by relative abundance. The features that passed the filter cut-off will be analyzed by statistical methods such as ANOVA, multiple testing correction by Benjamini-Hochberg, and fold change between diagnosis groups.

In the cases when the purpose is to classify a group of samples when prior information is unknown, unsupervised methods such as hierarchical clustering analysis, principal components analysis (PCA) or independent components analysis are the preferred methods. If the biomarkers in each diagnosis group are significantly different from each other, PCA can be used for visualization of the data in components of a multidimensional Figure on 2D or 3D plot that is easily understandable. PCA is also used in the reduction of data dimensionality and is helpful for investigating clustering tendencies (samples of a given diagnosis group tend to appear closer to samples of the same group), to detect outliers, and for visualization of data structure. PCA offers a simplified representation of the data contained in the spectrum, but it is not capable of analyzing additional information about the data, which is why PCA must be followed by a supervised analysis technique. [213, 228, 238]

In LC/MS statistical analysis, features commonly have small deviations in retention time from sample to sample as a consequence of column degradation, sample carryover, small fluctuations in room temperature or pressure and mobile phase pH, reloading mobile phase solvents, guard column filter degradation (which could lead to larger molecules passing the filter and causing interference), etc. This should be taken into account and corrected during statistical analysis.

Reduction of data “noise” caused by solvents or artifacts and contaminants that are present in the MS is a crucial step during data analysis in order to detect true features. Peaks in the chromatogram

need to be aligned and normalized in order to achieve reduction of noise and for an optimal data analysis process. Once the statistical analyses are completed and a list of statistically significant features (mass and retention time) is obtained, the identification of these features is the next step, which is crucial for determination of the biological relevance of the feature.

I.E.5.h. Feature identification. For identification of the features obtained by LC-MS, the accurate neutral mass or/and the mass to charge (*m/z*) is searched for in metabolite databases like Human Metabolome Database (HMDB), Metlin, InChI (International Chemical Identifier), Lipid Maps, ChEBI, Pubchem, Mass-Bank, Serum metabolome database (SMDB) and ChemSpider. These databases can facilitate metabolite identification and data interpretation. Currently none of the databases contains all of the known human metabolites. [211, 239]

Frequently a database search will yield more than one possible match for the queried compound; this complicates the identification of the selected candidate compound. A candidate compound is selected from the given list of potential compounds or hits given by the database. The selection should be based on the best mass accuracy (in parts per million) from the compounds in the list or it can also be selected based in its potential biological relevance. In order to reduce misidentifications, it is necessary to consider the isotope abundance information, which will lead to a smaller number of potential identifications. [240, 241]

After selecting a tentative identification of a feature from the databases, the identity of the feature must be confirmed by comparing the retention time and the MS/MS fragmentation patterns of a standard compound to that from the feature of interest in the experimental sample. This process is time intensive, and the respective standard may not be available for purchase or be cost prohibitive. Due to these reasons, MS/MS identification of features may become a rate-limiting step of an untargeted metabolomics experiment. [208] A different approach for identifying features in mass spectrum

obtained by MS/MS involves comparing the spectrum obtained with the specimen with spectrum libraries such as NIST (National Institute of Standards and Technology).

Frequently, potentially important metabolites detected in biological samples will not return matches from database searches. For these unknown metabolites, *de novo* identification is required. Nonetheless, it is important to recognize that comprehensive identification of all metabolite features detected in a metabolomics experiment is currently impractical for most samples. [207] The majority of metabolomics papers published to date are mostly limited to the first phase of biomarker discovery. This is because the interpretation of biomarker profiles is very difficult, and the formulation of hypotheses to explain the variations in metabolite profiles is difficult also. These pose great challenges for this field.[208]

I.E.5.i. Metabolomics standard initiative. A scientifically accepted context for reporting standardized metadata or results related to large-scale metabolomics data sets was created by the Metabolomics Standards Initiative (MSI). MSI defines the minimum reporting standards for metabolomics experiments. These include recommendations for sample preparation, experimental analysis, quality control, metabolite identification, and data processing. In addition, authors should report the level of identification of the metabolites reported in publications.[242, 243] There are four levels of identification:

- Level 1: Identified compounds (with chemical reference standards)
- Level 2: Putatively annotated compounds (without chemical reference standards, based on physicochemical properties and spectruml similarity with public/commercial spectrum libraries)
- Level 3: Putatively characterized compound classes. (Based on physicochemical characteristics or a chemical class of compounds or by spectruml similarity to known compounds).
- Level 4: Unknown compounds. Unidentified or unclassified compounds that still can be differentiated or quantified based in spectruml data.

I.E.5.j. Defining pathways. A metabolic profile is a disassembled puzzle of the cell´s activity. The understanding of how the molecules are produced, controlled, metabolized and excreted by the cell can be modeled and validated with the determination of the molecule´s pathway (chemical reactions occurring within a cell that modify the compound). The respective pathway usually can be described or at least hypothesized with proteomics and genomics analysis.[210]

There are publicly available, metabolic pathway databases that describe the metabolic pathway in which a certain feature will be either formed or assimilated. These databases include the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Cyc database, the reactome database, wikipathways, the small molecule pathway databases, and the Medical Biochemistry Page. Commercial databases available include TransPath, PathArt, MetaBase and Ingenuity Pathways Analysis. Some of these databases, like KEGG, have diagrams that can be very generic for describing a pathway leading to a specific metabolite. SMPDB (small molecule pathway database) is a pathway database focused on clinical biochemistry and pharmacology. SMPDB provides hand-drawn pathways that are specific to humans. These databases are being updated constantly.[211]

I.E.5.k. Machine learning algorithms for metabolite identification. Tandem-mass spectrometry metabolite identification is not always possible in metabolomics analysis. Identification requires comparison of the obtained spectrum against a database of reference spectra that must have been collected with similar equipment and acquisition parameters. These requisites are not always fulfilled, complicating identification of compounds that may not be present in the databases. A platform for prediction of a molecular spectrum using machine learning with the support vector algorithm can be used to aid in the prediction of the unknown compounds of the mass spectrum signals.[244] It is also used to predict properties to match against large molecular databases (Pubchem, MassBank). Studies have collected spectra from MassBank and compared and matched the predicted fingerprints against a large molecular database in order to obtain a list of potential identifications for the candidate

metabolites. The identification algorithm generalizes the metabolites that were not present in the reference spectra database and that were measured with different instrumentation or different acquisition methods. It was found that it is possible to determine the statistical dependencies between tandem mass spectra signals and molecular properties. This information can be used to classify metabolites with good identification performance. It was also concluded that a large enough set of fingerprint predictions can yield useful clues to the real metabolite identity to the "human expert", even if the exact automatic identification remains elusive. [244]

I.E.5.l. Lipidomics and eicosanomics. Specific platforms and approaches are available for specialized metabolomics studies. Some of the most provocative results presented in this dissertation involved lipid-related metabolites altered in DEN disease that will be discussed in detail in following chapters.

A description of the platforms devoted to study lipids and eicosanoids, termed lipidomics and eicosanomics respectively, follows:

i). *Lipidomics* is the research platform aimed at mapping the whole spectrum of lipids in a biological system. Lipidomics describes the function and metabolism of specific or individual molecules with the goal of understanding their roles in the composition of cellular membranes and their changes as consequences of diet, environmental stimuli, toxins, hormones, oxidants, pathogens, and pathobiochemical processes that are relevant to disease states.

Cells are able to synthesize hundreds of structurally diverse lipids; at least 300-400 different species of sphingolipids exist in the human body. Different types of cells will produce different types of lipids. The principal classes of lipid compounds are phospholipids, glycerols, sphingolipids, sterols and fatty acids. Lipids can be found compartmentalized into unevenly distributed biological structures.[245]

ii). *Eicosanomics* is the study of a very distinct group of lipid carboxylic acids called eicosanoids. The purpose of eicosanomics is to describe analytical strategies and methodologies in order to profile

the entire group of eicosanoids in biological systems using a mass spectrometry approach. The goal is to create, develop and apply methods to detect and quantify all the products from the oxidation of arachidonic acid in its respective pathways. Eicosanoids are the metabolic products of arachidonic acid oxidation, and there are at least 30 structurally different primary eicosanoids. More than 400 lipid-products are products of arachidonic acid metabolism. Major classes of eicosanoids include alcohols, hydroxyeicosatetraenoic acids (HETEs), epoxides, lipoxins, prostaglandins, prostacyclin, thromboxane and leukotrienes. Most of the eicosanoids are biologically potent lipids with functions in inflammation or immunity and are very unstable compounds. The eicosanoids can be found in subnanomolar concentrations and their main function is signaling through specific receptors. Eicosanoids have important roles in cardiovascular, renal, gastrointestinal and neuronal systems. Importantly, they are mediators of inflammation. [245]

The metabolic processes by which eicosanoids are synthesized involve oxidative pathways that incorporate oxygen into arachidonic acid (AA). Almost all cells can synthesize eicosanoids. Eicosanoids are created from enzymatic oxygenation by the enzymes cyclooxygenase (COX), lypoxygenase (LOX), and cytochrome P450 monooxygenase (MOX) isozymes. Isoeicosanoids and nitroeicosanoids are produced by a different pathway that involves free radical-mediated oxidations of AA. The pathways involved in the oxidation of AA can also oxidize other fatty acids including linoleic, linolenic and docohexaenoic acid.[245] Clotting stimulates blood cell eicosanoid biosynthesis, for this reason the level in serum of these metabolites does not reflect physiological concentrations. LC-MS analysis of these compounds is complicated because some isomers produce almost identical ESI/LC-MS-MS spectrum, for example prostaglandins E2 and D2.[245]

Lipidomics and eicosanomics are relevant to the investigation presented in further chapters, because along with metabolomics, these two platforms can be complementary in the study and understanding of lipids, fatty acids and compounds related to the host immune response. As mentioned

above, the pathways involved in the oxidation of AA are the ones that oxidize compounds that were found increased or with a positive FC in the results (Chapter 4) including α-linolenic acid and docohexaenoic acid, as well as lysophosphatidylcholines. More details on these compounds can be found on Chapter 3.

I.F. DISSERTATION OBJECTIVES

DEN is the most important arboviral disease; it is an important cause of morbidity in endemic countries, constantly overwhelming public health capacity. The development of new mosquito-based surveillance tests could provide early warning of impending epidemics and timely intervene to prevent human infections.

NS1 antigen detection in *Ae. agypti* was explored as a field-relevant technique for mosquito-based surveillance of DENV . The test proved to be sensitive and specific. A protocol for NS1 antigen detection in mosquitos was developed (Chapter 2).

The ability of NS1 antigen detection in urine and saliva to provide early noninvasive diagnosis of DEN infections was examined; non-invasive samples paired with serum from DEN infected patients were examined to provide early non-invasive diagnosis of DENV infections (Chapter 3).

The development of novel approaches to permit early identification of patients destined to progress to DHF/DSS would be of enormous value for public health practitioners and for clinicians managing patients. The ability to utilize non-invasive clinical specimens (e.g. – urine and saliva) for diagnosis and prognosis of DEN would also be of enormous value. Combining the two would potentially provide a true paradigm shift in DEN diagnosis.

The main goal of this dissertation research was to identify a biosignature panel of LC-MS/MS identified metabolites or candidate SMBS that differentiate the DEN disease diagnosis groups (DHF/DSS, DF, and ND) and that have potential for diagnosis and prognosis of DENV infections using acute phase serum and non-invasive clinical specimens (Chapter 4 and 5). Because it is unlikely that LC-MS/MS

technology will be applicable in the front line clinics where DEN patients first present, a long term goal of the research project is to select a subset of these pathogenically –and physiologically- relevant SMBs and then to determine the potential for the metabolite analyte or a surrogate (e.g., a protein involved in the metabolic pathway conditioning the metabolic change) to be incorporated into diagnostic formats amenable to point of care tests, such as ELISA based formats. A diagnostic algorithm incorporating results from such a POC test and conventional laboratory and clinical biomarkers could provide dramatically improved capability for diagnosis and prognosis of DENV infections and would be of immense value to physicians in managing patients.

The aims pursued in this dissertation are included in the following chapters:

Chapter 2. NS1 antigen detection in infected mosquitoes for mosquito-based surveillance for DENV.

Chapter 3. NS1 antigen detection in non-invasive (urine and saliva) specimens for DEN diagnosis.

Chapter 4. Metabolomics-based LC-MS detection of SMB's in serum and non-invasive samples for diagnosis and prognosis of DENV infections.

Chapter 5. Metabolomics-based tandem LC-MS identification of SMBs in serum and non-invasive clinical specimens (urine and saliva) for diagnosis and prognosis of DENV infections.

Chapter 6. Summary, conclusions and future directions.

CHAPTER 2

DETECTION OF DENGUE VIRUS NS1 ANTIGEN IN INFECTED AEDES AEGYPTI USING A COMMERCIALLY AVAILABLE KIT[1]

2.A. INTRODUCTION

Epidemic dengue has emerged throughout the tropical world. In the continued absence of a vaccine or treatment against dengue virus (DENV), mosquito vector surveillance and control programs are essential to reduce human infections. Dengue (DEN) is the most important mosquito-borne arboviral disease. The four dengue virus (DENV) serotypes (genus *Flavivirus,* family *Flaviviridae*) now circulate pan-tropically, [2, 41, 70, 99] and new genotypes associated with increased virulence have expanded from endemic areas of Asia into the Americas.[34, 35, 246, 247] Dramatic increases in epidemic dengue fever (DF) and dengue hemorrhagic fever (DHF) have occurred in recent decades, resulting in an estimated 100 million cases of DF, including 500,000 of DHF per year.[6] Although most cases occur in the endemic areas of the Americas, Southeast Asia, and the Pacific, DEN is a threat to the continental United States; DEN cases are diagnosed each year in travelers returning from endemic areas and autochthonous cases have been documented in Texas and recently also in Florida.[248-251] New and more effective tools and approaches for surveillance and control of DENV are sorely needed.[252]

In the absence of a vaccine, DENV prevention is focused upon controlling mosquito vectors. Development of improved surveillance methods for DENV in mosquito populations would be of great value for public health and vector control programs.[198, 252] For example, monitoring of mosquito populations for DENV infection could provide improved risk assessment for DENV infections in humans, and would allow vector control programs to better target their interventions to areas at greatest risk for ongoing or impending epidemics and to respond rapidly and more effectively to the emergence of DENV

[1] This chapter has been published in the American Journal of Tropical Medicine and Hygiene and is cited as: Voge, N.V., I. Sánchez-Vargas, C.D. Blair, L. Eisen, and B.J. Beaty. 2013. Detection of dengue virus NS1 antigen in infected Aedes aegypti using a commercially available kit. American Journal of Tropical Medicine and Hygiene 88: 260-266.

in new areas with susceptible human populations.[198] Although not typically used by DENV control programs, pathogen surveillance in vector populations is widely used for other arboviruses, for example West Nile virus.[253, 254] West Nile Virus surveillance in mosquitoes provides important spatial and temporal information about virus circulation and areas needing to be targeted for control and prevention efforts. In mosquito-based arbovirus surveillance, adult mosquitoes are typically collected using bait or oviposition traps, identified to sex and species, separated into pools that are assayed using one or a combination of tests, and field infection rates are then determined.[202, 253, 255-257] Because thousands of mosquitoes commonly are collected in surveillance programs, it is cost prohibitive to process individual mosquitoes, and pools of 100 or more mosquitoes are often tested to reduce costs.

DENV is maintained in mosquito-human transmission cycles; *Aedes aegypti* and *Aedes albopictus* are the most important vectors.[2] *Ae. aegypti*, the principal urban vector, lives in close contact with humans and commonly transmits the virus to humans in and around homes or other indoor environments.[73, 198] Mosquitoes become infected for life after feeding on a viremic human and, after a 4 -14 day extrinsic incubation period, the virus can be transmitted each time she bites susceptible humans.[71]

DENV can be detected in individual or pooled mosquitoes by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) for viral antigens, by reverse transcription-polymerase chain reaction (RT-PCR) for viral RNA, and by isolation of infectious virus.[257] However, DENV surveillance in mosquito vectors using these diagnostic techniques can be prohibitively expensive, may require special reagents, equipment or laboratory facilities or extensive training of personnel, and may be laborious and time consuming. An ideal test method for DENV surveillance in vectors would be simple to perform, rapid, inexpensive, cost-efficient, sensitive and specific, and capable of detecting the pathogen under field-relevant conditions. For example, the triturated suspensions of large pools of mosquitoes, which are viscous and contain particulates and environmental contaminants, can

complicate pathogen detection, especially by virus isolation and RT-PCR. In some circumstances, e.g., remote locations, mosquito traps may not be visited for extended periods of time, resulting in mosquito desiccation. In addition, mosquitoes may be subjected to cycles of freezing and thawing during identification, pooling, processing, and assaying the samples. All of these field-relevant conditions can result in infectious virus inactivation and/or destruction of viral analytes.[202]

RT-PCR is widely used for detection of arboviruses, including DENV, [73] in field-collected or simulated field-collected mosquito pools. RT-PCR has been demonstrated to detect one mosquito infected with Japanese encephalitis virus in a pool of 1,000 mosquitoes following 14 days of simulated tropical conditions.[201] DENV RNA can be detected in mosquitoes captured over a period of 28 days on sticky lure traps using RT-PCR,[258] and nested PCR has been shown to detect DENV in one infected mosquito head in pools of up to 59 negative mosquito heads.[259] Chikungunya virus RNA can be detected in *Ae. aegypti* mosquitoes stored at 28°C for 12 weeks.[260] Although an excellent test, RT-PCR is expensive and requires trained personnel, specialized equipment, and laboratory facilities.

Antigen detection systems using in-house ELISAs can be used for arbovirus surveillance in mosquitoes.[255, 256] Antigen detection kits are commercially available to detect WNV and Saint Louis encephalitis virus in mosquitoes.[254] Recently, Tan et al.[205] demonstrated that a commercially available ELISA kit designed to detect DENV nonstructural protein 1 (NS1) in human serum (Dengue NS1 Ag Strip®; Bio-Rad Laboratories, Marnes-la-Coquette, France) also could be used to detect DENV NS1 in infected *Ae. aegypti*. DENV NS1 antigen was detected in mosquitoes at 10 days after infection in the laboratory with DENV serotypes 1, 2, 3, or 4, as well as in field collected DENV-infected mosquitoes. The test was as sensitive as real-time RT-PCR in detecting DENV-infected mosquitoes.[205] Another NS1 test (Panbio® Dengue Early ELISA; Panbio, Brisbane, Australia) also proved to be sensitive for detection of DENV in experimentally-infected *Ae. aegypti* mosquitoes; NS1 was detected in pools of up to 50

mosquitoes at days 0, 5 and 15 post infection (PI). This portable test could be performed in 30 min, allowing for rapid monitoring of DENV in mosquitoes in the field.[261]

We expanded upon these studies and evaluated the utility of a third test - the Platelia™ Dengue NS1 Ag kit (Bio-Rad Laboratories, Marnes-la-Coquette, France) - to detect DENV NS1 antigen in mosquitoes subjected to conditions and processing steps commonly encountered in mosquito-based surveillance systems.[253, 255] We constructed pools of *Ae. aegypti* containing up to 999 uninfected mosquitoes and one infected mosquito subjected to simulated field conditions including drying (desiccation) and/or freeze-thaw cycles. The pools were then assayed for infectious DENV by virus isolation, for DENV RNA by RT-PCR and nested PCR, and for DENV NS1 antigen using the Platelia™ Dengue NS1 Ag kit.

2.B. MATERIALS AND METHODS

2.B.1. DENV preparation. To prepare virus for mosquito infections, C6/36 cells were cultured to confluent monolayers at 28°C using modified Eagle's medium (MEM) supplemented with 7% fetal bovine serum, L-glutamine, non-essential amino acids and penicillin/streptomycin. High passage DENV-2 (Jamaica 1409) was used to infect confluent monolayers at MOI 0.01. After seven days medium was replaced, and at 12-14 days medium was harvested and frozen at -70°C. Viral titer was determined by plaque assay.[262]

2.B.2. DENV titration by plaque assay. To titrate DENV, LLC-MK2 monkey kidney cells were grown to confluent monolayers in 12-well plates, infected with 10-fold serial dilutions of virus for 1 hour, then overlaid with an agar-nutrient mixture.[263] After 7 days of incubation at 37ºC cells were stained with 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution and incubated for 4 more hours.[262] Infectious viral titers were determined by counting plaques.

2.B.3. DENV infection of *Aedes aegypti* mosquitoes. *Ae. aegypti (*Puerto Rico Rex-D strain) eggs were hatched and reared to adults at 28° C/75-80% relative humidity with a photo-cycle of 16:8 L:D. To infect

mosquitoes, adult females were placed in cartons and deprived of sugar and water 4 hours before injection. Mosquitoes were intrathoracically injected with 1 x 10^3 to 1.5 x 10^3 PFU DENV-2 (Jamaica 1409) in a volume of ~0.5 µl. Mosquitoes were frozen at -70°C on days 1, 3, 7, 14 or 21 PI. To assay for DENV infection, individual mosquito heads were severed, squashed on a glass slide, fixed in acetone and assayed for DENV envelope protein antigen by indirect IFA, briefly; a 1:150 dilution of monoclonal antibody 4G2 in PBS was added to the head squashes and incubated for one hour at 37°C in humidified box. Then the slides were submerged in a tray with PBS for 5 minutes, and washings were repeated three times. After these washings, a second antibody consisting of goat anti mouse IgG-biotin (Sigma) diluted 1:200 in PBS and 1:200 of diluted Evans blue was added, and the slides were incubated for one hour. After washing three times as above, streptavidin-fluorescein diluted 1:200 was added and incubated for 20 minutes. Then the slides were washed twice as above with dH_2O. Diluted glycerin was added and cover-slides were placed before visualizing the slides in the microscope (Figure 14).[71] The corresponding mosquito bodies were kept at -70°C.

2.B.4. Construction and processing of mosquito pools. The bodies of mosquitoes injected with DENV were used to construct mosquito pools along with age-matched uninfected mosquitoes. Mosquitoes processed at 1 day PI did not contain detectable antigen in head tissues but were demonstrated by other assays to contain detectable DENV analytes (see Results section). Immunofluorescence assay of head-squash preparations of mosquitoes processed at 3-21 days PI revealed the presence of DENV antigens (Figure 14).

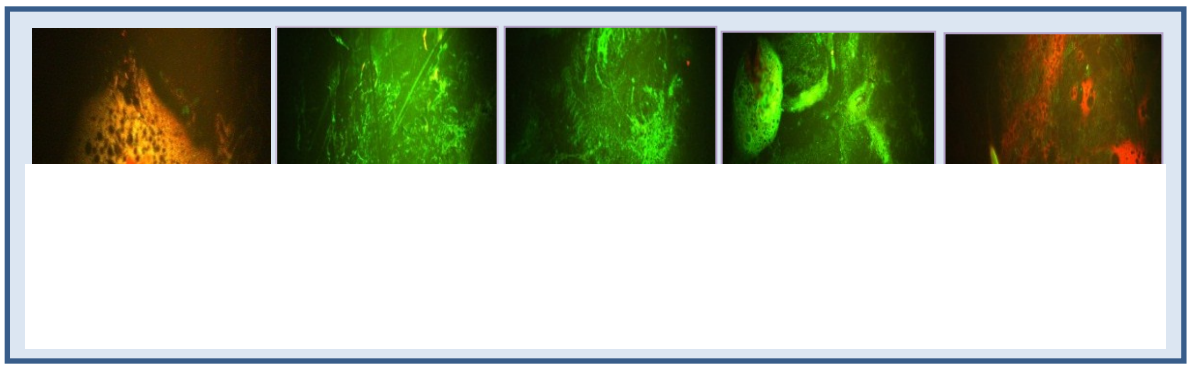


Figure 14. Selected IFAs of infected mosquito head-squashes. IFA assay. A. 1 day PI: B. 7 days PI; C. 14 days PI; D. 21 days PI; E. negative control.

Pools containing one DENV-infected and varying numbers of uninfected whole-mosquitoes (0, 9, 99, 499, or 999) were constructed for total pool sizes of 1, 10, 100, 500 or 1,000 mosquitoes. In order to address heterogeneity in virus loads likely to be encountered in field-collected mosquitoes due to differences in vector competence, times of extrinsic incubation, and environmental factors,[71, 264, 265] mosquitoes harvested 1, 3, 7, 14, or 21 days PI respectively were added to the pools of uninfected mosquitoes. Mosquito pools were triturated using cold pestles in 1.5 ml of L-15 medium for pools of 1 and 10 mosquitoes, 2 ml for pools of 100 and 500 mosquitoes, and 3 ml for pools of 1,000 mosquitoes. Medium (pH 7.2 $\pm$ 0.2) contained HEPES, FBS, L-glutamine, essential amino acids and penicillin-streptomycin. After trituration pools were centrifuged at 1,000 rpm for 10 min and supernatants were tested for NS1 Ag without further processing.

For experiments to simulate field conditions and processing, additional pools containing one infected mosquito and 0, 9, 99, or 999 uninfected mosquitoes were constructed. To address effects of freezing and thawing during processing, the constructed pools containing the bodies of uninfected mosquitoes to which one infected mosquito body was added were subjected to either 1 hour of freezing at -20°C and 1 hour of thawing at room temperature or five identical freeze-thaw cycles. To address effects of drying (desiccation) of mosquitoes, an additional set of pools was constructed with infected mosquitoes that had been maintained for 1, 3, 14, or 30 days at room temperature in a biosafety cabinet.

2.B.5. DENV NS1 antigen detection using the Platelia™ Dengue NS1 Ag kit. The Platelia™ Dengue NS1 Ag kit (Bio-Rad Laboratories, Marnes-la-Coquette France) was used to detect DENV NS1 antigen in mosquitoes. The test is a one-step sandwich format enzyme immunoassay for qualitative or semi-quantitative detection of DENV NS1 antigen from all four DENV serotypes.[200] Fifty µl of the supernatant of centrifuged mosquito homogenate suspensions were placed into each well of a 96-well plate and incubated with 150 µl anti-NS1 monoclonal antibody (MAb) conjugated with horseradish peroxidase in phosphate buffer, Tween 20, and fetal calf serum for 90 min at 37°C. When NS1 was present, an immune-complex MAb-NS1-MAb-peroxidase formed and was revealed by adding a chromogenic substrate (tetramethylbenzidine) and H_2O_2 to initiate color development. The reaction was stopped by the addition of 100 µl of 1N sulfuric acid. Absorbance was determined at 450 nm (A_{450}). Sample A_{450} values were compared to those of positive standards included in the kit.[200] Each sample was assayed in triplicate (Figure 15).

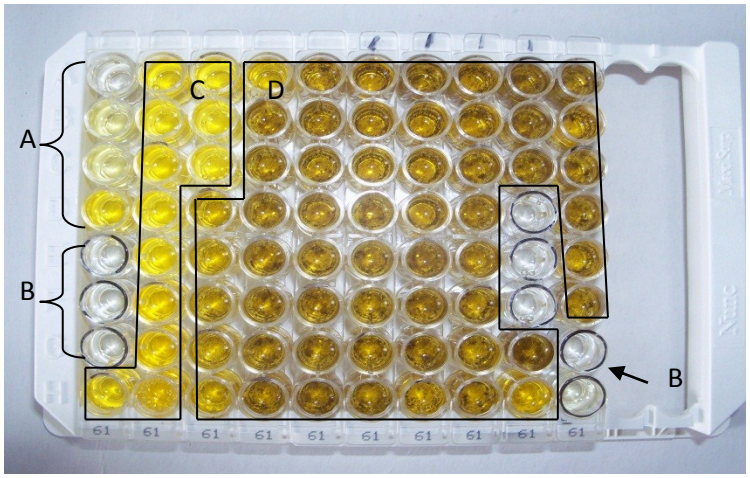


Figure 15. Example of ELISA plate for detection of NS1 antigen. Colored wells indicate the presence of NS1. A. Quality control standards provided by the kit. B. pools with 100 non-infected mosquitoes. C. wells with light yellow color had pools of <100 mosquitoes. D. Wells with darker yellow color had pools of ≥100 mosquitoes.

2.B.6. DENV RNA detection using RT-PCR and nested PCR. Mosquito pools were homogenized in L-15 medium, suspensions were centrifuged at 1,000 rpm for 10 min, supernatants were filtered sequentially through 0.45 µm and 0.20 µm diameter pore Acrodisc® HT Tuffryn membranes (Pall Corporation, Ann Arbor, MI) and total RNA was extracted from the filtrates using TRIzol LS (Invitrogen, California) following manufacturer recommendations. The RNA concentration and quality were determined for each sample by the 260/280 nm absorbance ratio using a Nanodrop Spectrophotometer (ND-100).

The RT-PCR was performed using the Qiagen One-step kit (Qiagen Inc. Santa Clarita, CA). The reaction mixture consisted of 100 ng (5µl) RNA, 5 µl 5X RT buffer, 1 µl dNTPs (10mM), 1 µl each forward and reverse primers (10µM), 1 µl enzymes (from the Qiagen mix containing Omniscript reverse transcriptase, Sensiscript reverse transcriptase and HotStarTaq DNA polymerase), 0.25 µl RNase inhibitor and RNase free water to a total volume of 25 µl. Primers were designed to amplify a 362 nt region of the DENV-2 NS3 gene: sense DV1 (GGRACKTCAGGW-TCTCC) and antisense DSP2 (CCGGTGTGCTCRGCYCTGAT).[266] Reverse transcription was performed at 52°C for 45 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 60 sec and 68°C for 60 sec, with a final extension at 72°C for 10 min. All samples and a positive control (consisting of extracted RNA from C6/36 cells infected with DENV-2 Jamaica 1409 virus) were analyzed by agarose gel electrophoresis followed by staining with ethidium-bromide and visualization using a UV transilluminator. To increase sensitivity, RT-PCR products were further amplified by nested PCR, the target size to amplify was 362 and the regions were NS3 (5279-5260).[259, 267] The reaction mixture consisted of 10 µl 2X PCR master mix (Promega, Madison, WI), 1 µl (10µM) each forward primer (5' AATTGTCGACAGAAAAGGAAA), and reverse primer (5' GGCTGGGGTTTGGTATC), 3 µl from the completed RT-PCR and H_2O to 20 µl. The protocol was 94°C for 2 min, then 30 cycles of 94°C for 30 sec and 55°C for 1 min, with an extension at 72°C for 10 min. Products were analyzed as above.

2.B.7. DENV detection in mosquito pools by virus isolation. Mosquito pools were homogenized, centrifuged and filtered as described above for DENV RNA preparation and filtrates were titrated by plaque assay as described above for DENV titration.

2.C. RESULTS

2.C.1. Detection of DENV in mosquito pools under ideal laboratory conditions. Mosquito pools were constructed using DENV-infected mosquitoes that had been processed using standard laboratory protocols. DENV-infected mosquitoes at 1 to 21 days PI were pooled with uninfected mosquitoes (0, 9, 99, 499, or 999), and the resulting pools of 1 to 1,000 mosquitoes were assayed for infectious DENV by plaque titration, for DENV RNA by RT-PCR/nested PCR, and for DENV NS1 antigen using the Platelia™ Dengue NS1 Ag kit (Tables 1 and 5).

Table 1. Effects of mosquito pool size and time after intrathoracic injection (day PI) on detection of DENV by virus isolation, RNA detection and NS1 antigen detection.

		Virus isolation	DENV-RNA		NS1
Pool size	Day PI	Titer (log_{10} PFU/ml)	RT-PCR	Nested PCR	Mean A_{450} (range)
1	1	1.94	positive	positive	5.18 (5.15-5.20)
1	7	4.34	positive	positive	6.35 (6.27-6.45)
1	14	4.72	positive	positive	6.17 (6.15-6.20)
1	21	3.54	positive	positive	6.08 (5.98-6.16)
1	Uninfected	negative	negative	negative	0.12 (0.12-0.12)
10	1	1.72	positive	positive	6.08 (5.92-6.08)
10	7	4.25	positive	positive	6.64 (6.27-7.35)
10	14	3.49	positive	positive	6.12 (6.07-6.20)
10	21	3.11	positive	positive	6.06 (6.06-6.06)
10	Uninfected	negative	negative	negative	0.15 (0.15-0.17)
100	1	ND	negative	positive	2.67 (2.24-2.98)
100	7	ND	positive	positive	6.25 (6.12-6.33)
100	14	ND	positive	positive	6.03 (6.02-6.06)
100	21	ND	positive	positive	6.17 (6.07-6.29)
100	Uninfected	negative	negative	negative	0.14 (0.14-0.14)
500	1	ND	positive	positive	3.61 (3.40-3.85)
500	7	ND	positive	positive	6.18 (6.11-6.23)

500	14	ND	positive	positive	6.01 (6.00-6.01)
500	21	ND	positive	positive	6.08 (5.97-6.17)
500	Uninfected	negative	negative	negative	0.17 (0.17-0.17)
1,000	1	ND	negative	negative	1.49 (1.24-1.49)
1,000	7	ND	negative	negative	3.07 (3.00-3.75)
1,000	14	ND	negative	negative	4.10 (4.09-4.10)
1,000	21	ND	negative	negative	6.65 (6.63-6.65)
1,000	Uninfected	negative	negative	negative	0.14 (0.14-0.14)

*Pools of 1 infected mosquito in the total pool size shown were assayed by virus titration, RT-PCR, nested PCR and NS1 Ag detection for DENV infection. ND = titer below level of detection, positive/negative = PCR product detected/not detected on gel.

Infectious DENV was isolated from pools containing 1 infected mosquito in a total of 1 and 10 mosquitoes; virus titers in the pools ranged from 1.72 $\log_{10}$ PFU/ml to 4.72 $\log_{10}$ PFU/ml (Table 1). The virus titers of given sized pools increased from day 1 to 7 PI and were highest in pools constructed with mosquitoes harvested at 7 and 14 days PI. DENV was below the level of detection by plaque assay in pools with $\geq$100 mosquitoes, regardless of the days PI of the infected mosquito in the pool (Table 1).

Mosquito pools were assayed by RT-PCR/nested PCR for the presence of DENV RNA. Amplicons of 362 bp for RT-PCR and 332 bp for nested PCR were detected in pools containing 1 to 500 mosquitoes regardless of the days PI of the DENV-infected mosquitoes (Table 1). In contrast, DENV RNA was not detected by either RT-PCR or nested PCR in pools containing 1,000 mosquitoes.

DENV NS1 antigen was detected both visually and spectrophotometrically using the Platelia™ Dengue NS1 Ag kit in all mosquito pools that had been prepared using ideal laboratory conditions (Table 1). The presence of NS1 was revealed by a yellow color, and the wells of the larger pools also contained a dark precipitate. A_{450} values above 1 are considered to be positive and indicate the presence of NS1. The mean A_{450} values of pools containing a single infected mosquito, harvested from 7 to 21 days PI, were similar for pool sizes ranging from 1 to 500 mosquitoes. In contrast to detection by virus isolation or RT-PCR/nested PCR, DENV NS1 antigen also could be detected in pools of 1,000 mosquitoes containing a single DENV-infected mosquito harvested from 1 to 21 days PI (Table 1). In pools with 1,000 mosquitoes, compared to those with fewer mosquitoes, A_{450} values were lower but still above the cut-

off threshold of 1. The mean A_{450} values of uninfected (negative control) pools ranged from 0.12 to 0.17. The range of A_{450} values in triplicate readings of both positive and negative pools was very small and consistent (Table 1).

All three tests (virus isolation, viral RNA detection by RT-PCR/nested PCR, and NS1 detection using the Platelia™ Dengue NS1 Ag kit) could be used to detect DENV in pools of 1 and 10 mosquitoes. False negative results were obtained in the virus isolation test for pools containing $\geq$100 mosquitoes and by RT-PCR/nested PCR for pools containing 1,000 mosquitoes. The Platelia™ Dengue NS1 Ag kit was the most sensitive test for detecting DENV in pools that contained infected specimens prepared under ideal laboratory protocols.

2.C.2. Detection of DENV in pools containing infected mosquitoes subjected to drying. To address the ability of the assays to detect DENV in mosquitoes in more field-relevant conditions, DENV-infected mosquitoes at 1, 7, 14, and 21 days PI were subjected to drying at room temperature for a further 1, 3, 14 or 30 days. The dried mosquitoes were used to construct pools of 1 infected in a total of 1, 10, and 100 mosquitoes, which were assayed for DENV by virus isolation, RT-PCR/nested PCR, and NS1 antigen (Tables 2 and 5).

Drying negatively affected DENV viability; only 10% (5/48) of the infected pools yielded virus isolates (Table 2). Virus isolation was most successful for small pool sizes and for a drying period not exceeding 3 days. However, the titers were low, ranging from 1.12 $\log_{10}$ PFU/ml to 1.94 $\log_{10}$ PFU/ml. Only a single virus isolate was made from a pool containing a mosquito at 14 days PI and dried for three days. Virus was not isolated from any pools containing DENV-infected mosquitoes at 1 or 21 days PI, or from any pools that contained DENV-infected mosquitoes that had dried for 14 or 30 days.

DENV RNA could be detected in most of the infected pools using RT-PCR and nested PCR tests, with the nested PCR being more sensitive for detecting DENV RNA in pools constructed with DENV-infected mosquitoes at 1 day PI (Tables 2 and 5). Overall, the sensitivities were 92% (44/48) for nested

PCR and 73% (35/48) for RT-PCR across drying conditions, due in part to false negative results in the RT-PCR test for pools constructed with mosquitoes at 1 day PI (Tables 2 and 5).

DENV NS1 antigen was detected in all but one pool with a total of 100 mosquitoes in which the infected mosquito had been subjected to 30 days of drying (borderline value of 0.94; Table 2) and one other pool that was subjected to the most extreme circumstances (pool size of 1,000, with the infected mosquito dried for 30 days and subjected to 5 freeze-thaw cycles; Table 4).

Only the mean A_{450} value is presented for each of the pools, because the range of values in triplicate samples varied minimally (see Table 1 for representative results). A_{450} values for pools containing mosquitoes at 7 to 21 days PI were consistently high regardless of the number of days dried (Table 2). The A_{450} values for pools constructed with mosquitoes at 1 day PI declined with the time of drying, notably in pools with 100 mosquitoes. It is likely that minimal virus replication has occurred and thus only a small amount of NS1 antigen has been produced in infected mosquitoes by 1 day PI or that NS1 antigen detected at 1 day PI was injected with the virus inoculum. In either case, the amount of NS1 antigen may be near the threshold of detection. The Platelia™ Dengue NS1 Ag kit proved to be the most sensitive test for detecting DENV in mosquito pools in which the infected mosquitoes had been subjected to drying, as could occur in field surveillance programs.

Table 2. Effects of drying infected mosquitoes on detection of DENV in mosquito pools by virus isolation, RNA detection and NS1 antigen detection.*

No. of days dried	Pool size	Virus Isolation Titer (log_{10} PFU/ml) by day PI					DENV- RNA Detection RT-PCR by day PI					DENV- RNA Detection Nested PCR by day PI					NS1 Ag detection Mean A_{450} by day PI				
		Un-infec-ted	1	7	14	21	Un-infec-ted	1	7	14	21	Un-infec-ted	1	7	14	21	Un-infec-ted	1	7	14	21
1	1	neg	0	1.94	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	6.88	6.96	6.92	6.73
1	10	neg	0	1.64	0	0	neg	pos	pos	pos	pos	neg	pos	pos	neg	pos	neg	6.90	6.92	6.85	6.62
1	100	neg	0	0	0	0	neg	neg	neg	pos	pos	neg	pos	pos	pos	neg	neg	6.33	6.91	6.88	6.70
3	1	neg	0	1.64	1.94	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	2.19	4.44	4.55	4.54
3	10	neg	0	1.12	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	4.66	4.61	4.56	4.53
3	100	neg	0	0	0	0	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	neg	4.08	4.60	4.54	4.57
14	1	neg	0	0	0	0	neg	neg	pos	pos	pos	neg	pos	pos	pos	pos	neg	1.33	5.98	5.66	4.70
14	10	neg	0	0	0	0	neg	neg	neg	pos	pos	neg	pos	pos	pos	pos	neg	1.82	5.71	5.55	4.68
14	100	neg	0	0	0	0	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	neg	1.16	5.62	5.54	4.69
30	1	neg	0	0	0	0	neg	neg	pos	neg	pos	neg	pos	pos	neg	pos	neg	1.44	6.27	6.16	6.11
30	10	neg	0	0	0	0	neg	neg	pos	pos	pos	neg	neg	pos	pos	pos	neg	2.06	6.12	6.12	6.00
30	100	neg	0	0	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	0.94	6.17	6.08	6.06

*****Mosquitoes at 1, 7, 14 or 21 days post-DENV infection (PI) were subjected to 1, 3, 14 or 30 days of drying at room temperature and pools of 1 infected mosquito in the total pool size shown were constructed and assayed by virus titration, RT-PCR/nested PCR or NS1 Ag detection for DENV infection. Pos = positive, neg = negative.

2.C.3. Detection of DENV in pools containing DENV-infected mosquitoes subjected to repeated freezing and thawing. To address the ability of the assays to detect DENV in mosquitoes in another potentially field-relevant situation, DENV-infected mosquitoes at 1, 7, 14, and 21 days PI were subjected to 1 or 5 cycles of 1 hour of freezing and 1 hour of thawing. The treated mosquitoes were used to construct pools of 1, 10, or 100 mosquitoes, which were assayed for DENV by virus isolation, RT-PCR/nested PCR, and NS1 antigen detection (Tables 3 and 5).

DENV was isolated from all pools (8/8) with a single infected mosquito subjected to 1 or 5 freeze-thaw cycles and from 88% of pools (7/8) with a total of 10 mosquitoes, the lone exception being a pool subjected to 5 freeze-thaw cycles and where the infected mosquito was harvested 21 days PI (Table 3). None of the pools containing 100 mosquitoes yielded a virus isolate. Overall, virus was isolated from 62% (15/24) of the pools containing DENV-infected mosquitoes. Virus titers were not reduced following multiple freeze-thaw cycles (Table 3). DENV RNA was detected in 96% (23/24) of mosquito pools by either RT-PCR or nested PCR following 1 or 5 freeze-thaw cycles (Table 3), demonstrating the stability of DENV RNA in the mosquito during freezing and thawing.

Table 3. Effects of freezing and thawing infected mosquitoes on detection of DENV in mosquito pools by virus isolation, RNA detection and NS1 antigen detection*

No. of freeze-thaw cycles	Pool size	DENV isolation Titer (log_{10} PFU/ml) by day PI					DENV RNA Detection RT-PCR by day PI					Nested PCR by day PI					DENV NS1 Ag detection Mean A_{450} by day PI				
		Uninfected	1	7	14	21	Uninfected	1	7	14	21	Uninfected	1	7	14	21	Uninfected	1	7	14	21
1	1	neg	1.94	4.49	3.64	3.64	neg	pos	pos	pos	pos	neg	pos	pos	pos	neg	neg	6.75	6.65	6.63	6.54
1	10	neg	1.00	4.25	3.64	3.11	neg	neg	pos	pos	pos	neg	neg	pos	neg	pos	neg	6.66	6.65	6.60	6.51
1	100	neg	0	0	0	0	neg	pos	pos	pos	pos	neg	pos	pos	neg	pos	neg	6.78	6.57	6.58	6.40
5	1	neg	1.94	4.41	3.64	2.94	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	neg	4.60	6.64	6.63	6.56
5	10	neg	1.64	4.11	3.64	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	6.38	6.55	6.65	6.55
5	100	neg	0	0	0	0	neg	pos	pos	pos	pos	neg	pos	pos	pos	pos	neg	4.94	6.57	6.60	6.56

*Mosquitoes at 1, 7, 14 or 21 days post-DENV infection (PI) were subjected to 1 or 5 freeze-thaw cycles and pools of 1 infected mosquito in the total pool size shown were constructed and assayed by virus titration, RT-PCR/nested PCR or NS1 Ag detection for DENV infection. pos = positive, neg = negative.

DENV NS1 antigen was detected by use of the Platelia™ Dengue NS1 Ag kit in every pool containing a DENV-infected mosquito (Table 3). Mean A_{450} values ranged from 4.60 to 6.78, all of which greatly exceeded the cut-off value. Multiple freeze-thaw cycles did not adversely affect NS1 antigen detectability.

Freezing and thawing samples had little effect on the ability to detect NS1 antigen in infected mosquito pools using the Platelia™ Dengue NS1 Ag kit nor the ability to detect DENV RNA by RT-PCR/nested PCR. Similarly there was little effect of freezing and thawing on DENV isolation when assaying mosquito pools of 1 or 10 mosquitoes (Table 3). The sensitivity of the Platelia™ Dengue NS1 Ag kit exceeded the sensitivity of virus isolation and slightly exceeded RT-PCR/nested PCR for detection of DENV in pools subjected to freeze-thaw cycles (Tables 3 and 5).

2.C.4. Detection of DENV in pools containing DENV-infected mosquitoes subjected to both drying and freezing-thawing. We also examined the ability of the assays to detect DENV in mosquitoes at 21 days PI that were subjected to a "worst case" field scenario of 5 cycles of freezing and thawing followed by 30 days of drying. The treated mosquitoes were used to construct pools of 1, 10, 100 and 1,000 mosquitoes, which were assayed for DENV by virus isolation, RT-PCR/nested PCR, and NS1 antigen ELISA (Tables 4 and 5). None of the pools yielded a virus isolate. In contrast, DENV RNA was detected by RT-PCR/nested PCR in pools containing up to 100 mosquitoes, but not in the one containing 1,000 mosquitoes. DENV NS1 antigen was detected using the Platelia™ Dengue NS1 Ag kit (Table 4) in similar pool sizes. Interestingly, the NS1 assay A_{450} values did not differ from those obtained with pools constructed with mosquitoes subjected to either drying or freezing-thawing alone (Tables 2 and 3), revealing the stability of the NS1 analyte under simulated field conditions.

Table 4 . Detection of infectious DENV, DENV RNA or NS1 antigen in mosquito pools for which the infected mosqutio had been subjected to both drying and freeze-thaw-cycles*

Pool size	Titer (log_{10} PFU/ml)	RT-PCR/ Nested PCR	Mean A_{450}
1	ND	pos/pos	6.39
10	ND	pos/pos	6.37
100	ND	pos/pos	6.33
1,000	n/a	neg/neg	Neg

*At 21 days post-DENV infection, mosquitoes were subjected to 30 days of drying at room temperature followed by 5 cycles of freezing and thawing. Pools of 1 infected and treated mosquito in the total pool size shown were constructed and assayed by virus titration, RT-PCR/nested PCR or NS1 Ag detection for DENV infection. ND = titer below the level of detection, n/a = pool not assayed, pos = positive, neg = negative.

A summary of the sensitivities obtained with each assay used for testing the detection of NS1 antigen can be found in Table 5. The NS1 Ag kit was 100% sensitivity under normal laboratory conditions, while nested PCR and RT-PCR where 80% and 75% respectively. Virus isolation was positive only in 40% of the pools. The results obtained when the pools were dried or freeze-thawed, also showed that NS1 has a higher sensitivity than the other tests (Table 5).

Table 5 . Comparison of test sensitivities under the assayed conditions.*

	Percentage of positive pools (no. positive/examined) in relation to the conditions to which DENV-infected mosquitoes contained in the pools were exposed				
Test method	Normal laboratory conditions	1-30 days of drying	1-5 freeze-thaw cycles	30 days of drying and 5 freeze-thaw cycles	Grand total for all conditions
Virus isolation	40% (8/20)	10% (5/48)	62% (15/24)	0% (0/3)	29% (28/95)
RT-PCR	75% (15/20)	75% (36/48)	92% (22/24)	75% (3/4)	79% (76/96)
Nested PCR	80% (16/20)	92% (44/48)	83% (20/24)	75% (3/4)	86% (83/96)
NS1 Ag kit	100% (20/20)	98% (47/48)	100% (24/24)	75% (3/4)	98% (94/96)

*A summary of the sensitivities obtained in each assay, including DENV-infected mosquitoes subjected to normal laboratory conditions, drying (desiccation), freeze-thaw cycles, or a combination of drying and freeze-thaw cycles. These summary data include all tested pools from Tables 1-4.

2.D. DISCUSSION

Our findings strongly suggest that NS1 antigen detection is a promising approach for DENV surveillance in vector mosquitoes and confirm previous studies in this regard. We were able to detect DENV NS1 antigen by use of the Platelia™ Dengue NS1 Ag kit in pools containing one infected mosquito and up to 999 uninfected mosquitoes, as well as in pools constructed with infected mosquitoes that had been subjected to field-relevant handling conditions including drying for up to 30 days and/or multiple cycles of freezing and thawing. Overall, detection of DENV infection by use of the Platelia™ Dengue NS1 Ag kit was more sensitive than RT-PCR and virus isolation in our laboratory and simulated field condition-pools of mosquitoes (Table 5).

Virus isolation was the least sensitive test for detection of DENV in pools containing ≥100 mosquitoes and in pools that had been subjected to field relevant collecting and processing conditions. The overall sensitivity of virus isolation for detection of DENV in the infected pools assayed was 29%. The RT-PCR and nested PCR assays yielded overall sensitivities of 79 and 86%, respectively. The overall sensitivity of the Platelia™ Dengue NS1 Ag kit ELISA was 98%; the only false negative results were obtained with one pool with an A_{450} value of 0.94, which is borderline positive, and one pool of 1,000 mosquitoes for which the infected mosquito had been subjected to 30 days of drying and 5 cycles of freezing-thawing (Tables 2 and 4). Detection of DENV NS1 antigen by use of the Platelia™ Dengue NS1 Ag kit was more efficacious than detection of DENV RNA by RT-PCR/nested PCR in very large pools and in pools that had been subjected to simulated field conditions, especially drying of mosquitoes (Table 2).

The false negative results with the RT-PCR/nested PCR tests may be attributable to problems in extracting the RNA analyte from homogenates of the concentrated mosquito suspensions in the larger pools and from dried tissues in mosquitoes subjected to drying. Similarly, virus isolation was compromised by large pool sizes (Tables 1, 2 and 3). There might have been proteases and/or RNases in the concentrated mosquito suspensions that could inactivate virus or degrade RNA. We included fetal

bovine serum and HEPES buffer in the trituration diluent to inhibit trypsin and other protease activity and to preserve virus integrity, but we did not add RNase inhibitors during homogenization. Very importantly for mosquito based arbovirus surveillance, sensitivity of NS1 antigen detection was not compromised in the larger pools (Tables 1-4).

We infected the mosquitoes by intrathoracic inoculation. While this had the advantage of providing mosquitoes of known infection status and virus titer, mosquitoes collected in the field will be infected orally and will likely exhibit variable virus titers and analyte accumulation due to differences in time of extrinsic incubation, permissiveness to DENV infection and replication, and environmental conditions including temperature.[71, 264, 265] Viral tissue tropisms and load also may be different in intrathoracically infected mosquitoes as compared to orally infected mosquitoes and could affect the efficacy of the respective tests. However, the previous study by Tan et al.[205], which used Bio-Rad's Dengue NS1 Ag Strip® test, demonstrated that detection of DENV NS1 antigen was successful in orally infected as well as field collected specimens. Interestingly, in our studies DENV NS1 could be detected using the Platelia™ NS1 Ag kit in nearly all of pools that tested false negative by RT-PCR/nested PCR. The role of virus load in these results needs to be investigated in both laboratory and field studies.

We determined the sensitivity of the Platelia™ Dengue NS1 Ag tests using only DENV-2. Similar studies need to be conducted with DENV-1, -3, and -4. The monoclonal antibody used in the test has been demonstrated to detect the NS1 protein of all 4 DENV serotypes, albeit with some differences in sensitivity.[268] Moreover, Tan et al.[205] demonstrated that all 4 DENV serotypes could be readily detected in infected mosquitoes using the Dengue NS1 Ag Strip® test from the same manufacturer as the Platelia™ Dengue NS1 kit. Nonetheless it would be prudent to determine the ability to detect DENV-1, -3, and -4 NS1 in mosquitoes subjected to the simulated field collection conditions by use of the Platelia™ Dengue NS1 Ag kit. In this regard, a new NS1 detection kit that differentiates DENV serotypes and that has excellent sensitivity and specificity has been developed using serotype-specific anti-NS1

monoclonal antibodies.[269, 270] In future studies, we will determine the diagnostic efficacy of the Platelia™ Dengue NS1 Ag kit for surveillance for DENV in vector populations in endemic areas and will address the issues of detection of the NS1 antigen in field-collected mosquitoes infected with different DENV serotypes.

In summary, our studies suggest that NS1 is an excellent analyte for DENV surveillance in mosquito vectors. Detection of DENV-infected mosquitoes with the Platelia™ Dengue NS1 Ag kit was more sensitive than either virus isolation or RT-PCR/nested PCR. Clearly, the Platelia™ Dengue NS1 Ag kit or other NS1 assays could be fruitfully applied to DENV surveillance in *Ae. aegypti* and could become a significant addition to the armamentarium of vector surveillance and control programs as previously suggested by Tan et al.[205] and Muller et al.[261] The Platelia™ Dengue NS1 Ag kit proved to be a simpler, more rapid, and less laborious test for detection of DENV-infected mosquitoes than either virus isolation or RT-PCR/nested PCR. Theoretically, the Platelia™ Dengue NS1 Ag kit could be more cost effective than the other tests because up to 92,000 mosquitoes could be tested with a single plate using pools of 1,000. A cost analysis comparing the three types of tests would be most informative in this regard.[271] Importantly, the Platelia™ Dengue NS1 Ag kit could be readily and rapidly applied by public health agencies not versed in mosquito-based arbovirus surveillance in the face of emergence of DENV in new geographic areas such as in the southern U.S. or southern Europe.

CHAPTER 3

DENGUE VIRUS NS1 ANTIGEN DETECTION IN SERUM AND NON-INVASIVE CLINICAL SPECIMENS FROM DENGUE PATIENTS

3. A. INTRODUCTION

Dengue (DEN) diagnosis is still a challenging problem in the developing world.[41] Serum is not an ideal specimen for diagnostic tests because of the reluctance of patients (especially children) to be bled, the difficulty in obtaining blood from very young children, the need for trained personnel and sterile equipment to procure the samples, and the potential for adverse events to occur. Non-invasive clinical specimens, such as saliva and urine, preclude expenses and difficulties associated with venipuncture, are readily and inexpensively collected, and do not engender resistance in patients or their guardians to provide the samples. Currently, there is no accepted test to diagnose dengue virus (DENV) infections using non-invasive clinical specimens. However, there are a number of reports of detection of DENV analytes in non-invasive clinical specimens.[163, 165-167]

DENV NS1 antigen detection tests have been commercially developed and are available for point of care diagnosis with serum specimens. The NS1 protein has a molecular weight of 46-55kDa. This cell-membrane-associated protein can be located in vesicular compartments within the cell, or on the cell surface as a secreted hexamer.[39, 54, 272, 273] The development of a NS1 diagnostic test, first by Young et al,[272] improved the diagnosis of DEN because it provides a relatively low cost, fast and simple test that is both sensitive and specific for serum samples.[55] However, the test has not been evaluated systematically for detection of NS1 in non-invasive clinical specimens. NS1 antigen capture ELISA also was successfully adapted for detection of antigen in DENV-infected mosquitoes (Chapter 2).[204] NS1 can be detected effectively in serum in primary and secondary infections, and the limits of antigen that can be detected are 15 µg/ml in acute phase sera.[272]

There are at least two case reports of DENV RNA detection in urine specimens.[165, 167] However, there have been no studies to investigate the potential of this approach for diagnostic testing. More effort has been devoted to detection of DENV-specific immunoglobulins (Igs) in urine; specific Igs have been detected in both primary and secondary infections.[168] IgG was detected 6 days post symptom onset, but the sensitivity was low (61%).[168] DENV-specific IgA was also detected in urine of patients, but only in secondary cases.[168] The potential for detection of NS1 in urine remains to be determined. With the exception of albumin, which has a diameter of ~7 nm, urine from healthy subjects normally lacks proteins. The NS1 protein has an estimated diameter of ~10 nm. [273] The similar sizes of these proteins suggest that NS1 may also be able to pass through the kidney and be detected in urine. Urine has an acidic nature, which could produce denaturation of protein analytes. Nonetheless, the NS1 hexamer has been shown to be resistant in high molarities,[273] therefore presumably it would be stable in an acidic environment such as urine.

Saliva has also been investigated as a potential clinical specimen for DEN diagnosis. DENV-IgM, IgG and IgA have been detected in saliva from DEN patients,[163, 168] with IgG detected only in secondary infections.[168] However, the reported sensitivity of this approach was not satisfactory, and these tests are not currently recommended for DEN diagnosis. Oral swabs from DEN patients have been analyzed for IgM and IgG as well as DENV NS1 antigen. Again, the sensitivity of the assays was low when compared to serum-based assays.[169] Currently, no test using non-invasive samples has been successfully developed for DEN diagnosis. In contrast to urine, saliva contains an abundance of proteins, suggesting that NS1 may also be detectable in this fluid. However, saliva is a notoriously viscous liquid which could complicate detection of specific analytes.

In this chapter, the potential for diagnosing human DENV infections using NS1 antigen detection in acute phase urine and saliva samples was investigated. Urine and saliva differ dramatically from serum in many ways, and these differences could condition the diagnostic efficacy of the sample for

diagnosis. The metabolomics studies (Chapters 4 and 5) of non-invasive clinical samples (saliva and urine specimens) from dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), dengue fever (DF) and non-dengue (ND) patients included assaying the respective samples for the presence of DENV analytes, specifically the presence of DENV NS1 protein. This provided a unique opportunity to investigate the diagnostic efficacy of detection of DENV NS1 protein in saliva and urine for diagnosis of DENV infections.

In this pilot study, NS1 detection rates in linked serum and non-invasive samples were determined and compared. NS1 was detected in urine and saliva samples from DEN patients, but the sensitivity was low in both acute and convalescent phase specimens from DHF/DSS as well as DF patients.

3.B. MATERIALS AND METHODS

3.B.1. Clinical samples. Acute and convalescent phase samples from febrile patients, including paired and non-paired serum, urine and saliva samples, were collected from patients in Mexico. The samples were collected from patients who presented with a febrile DEN-like disease, and who were referred to the laboratory clinic of the Universidad Autónoma de Yucatán - Centro de Investigaciones Regionales Dr. Hideyo Noguchi by a primary-care physician. The patients were interviewed, and serum, urine and saliva specimens were collected. These samples were procured as part of the normal DEN diagnosis mission of the laboratory clinic, and not as part of an experimental protocol. Patients were of both sexes and different age groups. Serum samples were collected with an aseptic technique, using sterile syringes; sample aliquots were stored in micro-centrifuge tubes that were promptly frozen. Urine was collected in sterile cups and saliva in conical tubes; both specimens were aliquoted and frozen promptly after being collected. The serum clinical samples were then tested in Mexico, by trained personnel in the same facility where they were collected, for IgM or DENV-RNA by ELISA or RT-PCR respectively. Positive serum

samples were then classified into DHF/DSS, DF or ND by a clinician based on the patient's clinical signs and symptoms.

Anonymous linked serum, saliva, and urine specimens (when available) were then sent to CSU for metabolomics and NS1 analysis. These studies were approved by the Institutional Review Board of Colorado State University and the Bioethics Committee of Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán

Urine and saliva samples were thawed on ice and aliquoted. Vials were centrifuged at 14,000 rpm, 4°C, for 15 minutes. 50 µl of each specimen were aliquoted into new vials and assayed for the presence of DENV NS1 antigen.

3.B.2. NS1 antigen detection using the Platelia™ Dengue NS1 Ag kit. The Platelia™ Dengue NS1 Ag kit (Bio-Rad Laboratories, Marnes-la-Coquette France; Catalogue No.72830) was used to test for the presence of NS1 antigen in clinical specimens. The test kit is a one-step sandwich format enzyme immunoassay for qualitative or semi-quantitative detection of DENV NS1 antigen of all four DENV serotypes. 50 µl of urine, saliva or serum were placed into each well of a 96-well plate and incubated with 150 µl anti-NS1 monoclonal antibody (MAb) conjugated with horseradish peroxidase in phosphate buffer, Tween 20, and fetal calf serum for 90 min at 37°C. When NS1 was present, an immune-complex MAb-NS1-MAb-peroxidase formed and was revealed by adding a chromogenic substrate (tetramethylbenzidine) and H_2O_2 to initiate color development (Figure 16). The reaction was stopped by the addition of 100 µl of 1N sulfuric acid. Absorbance was determined at 450 nm (A_{450}). Sample A_{450} values were compared to those of positive standards included in the kit. The interpretation of results was based on guidelines provided by the manufacturer; an OD reading of $\geq$ 1 was considered positive.

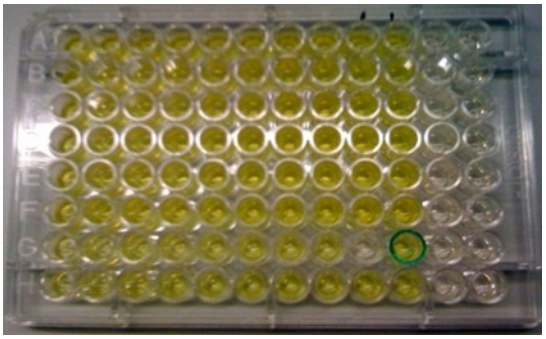


Figure 16. Example of NS1 antigen detection test. In this plate, the first 10 columns contained positive (DHF/DSS/DF) serum samples, the last two columns contained negative samples. The wells with a yellow color reveal presence of NS1 protein, while the wells that are almost transparent indicate a negative result.

3. C. RESULTS

A total of 34 serum (22 acute, 12 convalescent), 164 urine (116 acute and 48 convalescent) and 169 saliva (105 acute and 64 convalescent) samples from patients with known DHF/DSS, DF or ND classification were assayed for DENV NS1 antigen (Table 6). From spectrophotometric analysis, A_{450}values $\geq$1 were considered to be positive by manufacturer instructions and indicative of presence of NS1. A_{450}values ranged from 0.02 in ND samples to 20.84 for one DHF sample; the latter result was an outlier. Serum yielded the highest A_{450} values in both acute and convalescent phase samples. Six of the serum samples had A_{450} values that ranged from 9.64 to 11.1.

Data for the sensitivity of the kit for detection of NS1 in each type of specimen in the acute or convalescent phase, by diagnosis classification, are presented in Table 6. As expected, the acute phase specimens typically had a higher prevalence of NS1-positive samples than the convalescent ones. Positive acute phase serum samples were recorded for 2 of 4 (50%) DHF/DSS cases and 5 of 13 (38.4%) DF cases. For acute phase urine samples, 6 of 18 (33.3%) DHF/DSS cases and 10 of 72 DF cases (13.8%) tested positive for NS1. The corresponding numbers for acute phase saliva samples testing positive for NS1 were 5 of 14 (35.7%) for DHF/DSS cases and 18 of 69 (26%) for DF cases. NS1 antigen also was

detected in one saliva sample from a ND patient. (Table 6) This false positive result may have been due to the viscosity of the saliva sample.

Table 6 . Summary of test sensitivity for the types of specimen tested, broken down by acute and convalescent phase and by diagnosis classification.*

	Percentage of NS1 positives (no. positives/no. examined samples) for acute phase samples taken ≤5 days after onset of symptoms			Percentage of NS1 positives (no. positives/no. examined samples) for convalescent phase samples taken ≥6 days after onset of symptoms		
	DHF/DSS	**DF**	**ND**	**DHF/DSS**	**DF**	**ND**
Serum	50.0% (2/4)	38.4% (5/13)	0 (0/5)	0 (0/2)	66.7% (4/6)	0 (0/4)
Urine	33.3% (6/18)	13.8% (10/72)	0 (0/26)	7.6% (1/13)	5.2% (1/19)	0 (0/16)
Saliva	35.7% (5/14)	26.1% (18/69)	4.5% (1/22)	7.1% (1/14)	18.5% (5/27)	0 (0/23)

*Table includes, for each type of specimen, the number of NS1 positive samples over the total number of samples examined, and the sensitivity for each diagnosis classification. DHF/DSS: dengue hemorrhagic fever/dengue shock syndrome, DF: dengue fever, ND: non-dengue.

NS1 antigen was also detected in convalescent phase samples (Table 6). For convalescent phase serum samples, NS1 antigen was detected in 4 of 6 specimens (66.7%) of DF patients but was not detected in DHF/DSS cases serum of convalescent phase. Convalescent phase urine samples were NS1 positive for 1 of 13 (7.6%) DHF/DSS cases and 1 of 19 (5.2%) DF cases. For saliva, only 1 of 14 (7.1%) DHF/DSS cases and 5 of 27 (18.5%) DF cases tested positive for NS1. In the convalescent phase samples, there were no false positive results (Table 6).

The potential for the NS1 test to differentiate DEN (DHF/DSS and DF) cases from ND cases was determined in DEN diseased individuals compared to ND. NS1 detection rates in acute phase specimens were determined and compared (Table 7). NS1 was detected in 41.2% of serum specimens from DEN patients, 17.77% of urine specimens and 27.71% of saliva specimens. From ND patients, only one saliva specimen tested positive.

The potential for the NS1 test to differentiate DEN (DHF/DSS and DF) cases from ND cases was determined in DEN diseased individuals compared to ND. NS1 detection rates in acute phase specimens were determined and compared (Table 7). NS1 was detected in 41.2% of serum specimens from DEN patients, 17.8% of urine specimens and 27.7% of saliva specimens. From ND patients, only one saliva specimen tested positive.

We also compared statistically the likelihood of detecting NS1 for DEN disease cases based on examination of serum versus urine or saliva (Table 7). The likelihood of detecting NS1 antigen from DEN cases was similar for saliva versus urine samples (P=0.12) and for serum versus saliva samples (P=0.28). However, it was significantly higher for serum versus urine (P=0.04).

Table 7 . Detection of NS1 antigen in acute phase specimens (≤5 days after symptom onset) for serum and non-invasive samples*

	Percentage of positives (no. positive/examined)	
	DEN disease	**Non-DEN disease**
Serum	41.2% (7/17)	0 (0/5)
Urine	17.77% (16/90)	0 (0/26)
Saliva	27.71% (23/83)	4.5% (1/22)

*Table includes, for each type of specimen, the number of NS1 positive samples over the total number of samples examined, and the sensitivity for each diagnosis classification.

The concordance of diagnostic results in linked specimens from a given patient also was determined using samples from 30 patients that provided serum, urine, and saliva paired specimens. These included 18 patients diagnosed with DF, 5 with DHF/DSS, and 7 classified as ND. From this total of 30 patients, only two tested positive for NS1 in serum, urine and saliva. One of these patients was diagnosed with DHF/DSS, with samples collected two days after onset of symptoms. The other patient was diagnosed with DF, and samples were collected six days after onset of symptoms. In summary, just one patient with acute phase linked samples (serum, urine and saliva) tested NS1 positive, and one additional

patient with convalescent phase linked samples (serum, urine, saliva) tested positive. There was complete concordance across specimen types for only 6.7% of the linked samples (2/30).

An additional 109 paired urine and saliva samples were tested for concordance (serum was not included because the test requires 100 µl for the analysis and there was insufficient volume). Among this group, 57 paired samples were from DF patients, 25 paired samples from DHF/DSS patients, and 27 paired samples from ND patients (data not shown). Of these, 10 had concordant positive results; seven from DF patients and three from DHF/DSS patients. Seven of these were acute phase DF samples; two were acute phase DHF/DSS samples, and the last one a DHF/DSS convalescent sample. Overall, for urine and saliva paired samples, only 9.2% (10 of 109 cases) showed concordance.

The average A_{450} values differed among serum and non-invasive acute phase DENV-infected samples (Table 8). For acute phase serum specimens, the A_{450} values ranged from 0.03 to 10.94 for DHF/DSS patients and from 0.17 to 10.87 for DF patients. If only the positives for NS1 are considered, then the A_{450} values in serum ranged from 10.91 to 10.93 for DHF/DSS specimens and from 4.07 to 10.87 for DF specimens (Table 8).

Table 8 . NS1 antigen detection absorbance values in acute phase specimens (≤5 days after symptom onset) for serum and non-invasive samples in different diagnosis groups*

	Mean A_{450} (range) for all acute phase samples			Mean A_{450} (range) for only NS1 positive acute phase samples		
	DHF/DSS	**DF**	**ND**	**DHF/DSS**	**DF**	**ND**
Serum	7.41 (0.03-10.94)	2.98 (0.17-10.87)	0.28 (0.06-0.65)	10.92 (10.91-10.93)	7.32 (4.07-10.87)	-
Urine	1.01 (0.09-20.84)	0.54 (0.07-7.09)	0.14 (0.07-0.41)	2.97 (0.99-20.84)	2.49 (1.04-7.09)	-
Saliva	1.48 (0.15-10.04)	0.90 (0.02-6.67)	0.35 (0.12-2.23)	4.54 (1.04-10.00)	2.50 (1.00-6.67)	2.23 (2.23)

*Table presents mean optic density readings for each diagnosis classification and each kind of specimen type, and results are classified as acute and convalescent samples. DHF/DSS: dengue hemorrhagic fever/dengue shock syndrome, DF: dengue fever, ND: non-dengue. The cells that instead of a range have only the same value as the "mean" had only one positive sample.

In acute phase urine samples, the A_{450} values ranged from 0.09 to 20.84 for DHF/DSS patients and from 0.07-7.09 for DF patients. If only the positives for NS1 are considered, then the A_{450} values in

urine ranged from 0.99-20.84 for DHF/DSS patients and from 1.04-7.09 for DF patients (Table 7). In acute phase saliva samples, the A_{450} values ranged from 0.15 to 10.04 for DHF/DSS patients and from 0.02-6.67 for DF patients. If only the positives for NS1 are considered, then the A_{450} values in saliva ranged from 1.04 to 10.00 in DHF/DSS patients and from 1 to 6.67 for DF patients (Table 7). One sample from a patient diagnosed as ND yielded a positive value of 2.23; this was the only ND case that tested positive.

The average A_{450} values differed among convalescent phase samples for DENV-infected patients (Table 9). In convalescent phase serum specimens, the A_{450} values ranged from 0.06 to 0.22 for DHF/DSS patients and from 0.20 to 11.1 for DF patients. If only the positives for NS1 are considered, then the A_{450} values in serum ranged from 1.11 to 11.1 for DF patients (Table 9).

Table 9 . NS1 antigen detection absorbance values in convalescent phase specimens (≥6 days after symptom onset) for serum and non-invasive samples*

	Mean A_{450} (range) for all convalescent samples			**Mean A_{450} (range) for only NS1 positive convalescent samples**		
	DHF/DSS	**DF**	**ND**	**DHF/DSS**	**DF**	**ND**
Serum	0.14 (0.06-0.22)	5.50 (0.20-11.10)	0.37 (0.23-0.65)	-	8.11 (1.1-11.1)	0.37 (0.23-0.65
Urine	0.25 (0.07-1.29)	0.76 (0.76-10.10)	0.19 (0.10-0.44)	1.29 (1.29)	10.10 (10.10)	-
Saliva	0.38 (0.10-2.82)	1.22 (0.14-10.94)	0.22 (0.10-0.56)	2.82 (2.82)	10.94 (10.94)	-

*Table presents mean optic density readings for each diagnosis classification and each kind of specimen type, and is classified among acute and convalescent samples. DHF/DSS: dengue hemorrhagic fever/dengue shock syndrome, DF: dengue fever, ND: non-dengue. The cells that instead of a range have only the same value as the "mean" had only one positive sample.

In convalescent phase urine samples, the A_{450} values ranged from 0.07 to 1.29 for DHF/DSS patients and from 0.76 to 10.1 for DF patients. If only the positives for NS1 are considered, then the A_{450} values for urine consisted of just one sample (1.29) from a DHF/DSS patient and one sample (10.10) from a DF patient (Table 9). In convalescent phase saliva samples, the A_{450} values ranged from 0.10-2.82 for DHF/DSS patients and from 0.14 to 10.94 for DF patients. If only the positives for NS1 are considered, then the A_{450} values for saliva were 2.82 for one DHF/DSS patient and 10.94 for one DF patient (Table 9).

The acute phase serum, urine and saliva specimens of DHF/DSS yielded higher mean A_{450} readings compared to the convalescent phase ones. In DF specimens, the convalescent-phase samples had higher values in serum, urine and saliva (Table 8 and 9). In non-invasive samples, the average absorbance values did not vary substantially among acute and convalescent phase samples or by diagnosis group (Table 7 and 9).

3.D. DISCUSSION

The Platelia™ Dengue NS1 Ag kit test was designed to assay for NS1 antigen in serum specimens. The test has proven to be sensitive and indeed is now routinely used in many diagnostic laboratories for diagnosis of DENV infections. [166, 200, 268, 274-278] It is recognized that that the sensitivity of the test is lower when assaying serum specimens from patients experiencing secondary DENV infections. [55]. Unfortunately, in this limited study no information was provided concerning whether or not the patients were experiencing primary or secondary infection. In any future studies, this issue will need to be addressed. Secondary infections among the tested DEN patients could explain why the percentages of NS1-positive acute phase serum samples in this study (38.4% for DF patients and 50.0% for DHF/DSS patients) were lower than reported in other studies. [268, 274, 275, 279] In a study performed with samples from the *Institut Pasteur de la Guyane* the Platelia kit showed a sensitivity of 87.4%,[274] and a study in Puerto Rico revealed a sensitivity of 83.2% in acute serum samples [275]. Moreover, a multicountry study in Asia and the Americas revealed a sensitivity of 66% for the Platelia test in acute phase serum specimens.[268]

Chuansumrit et al. [4] also investigated the potential for diagnosis of DENV infections by detection of NS1 antigen in urine. These investigators used a standard ELISA method instead of a commercial test. The reported detection of NS1 antigen in urine samples was 68% for DF and 63% for DHF. In that study, the investigators compared the sensitivity of their in-house ELISA test to that of the commercially available NS1 strip test. The strip test showed a sensitivity of 52% for DF and 47% for DHF, lower than

that of the in-house ELISA method developed.[166] In the study with the Platelia kit presented herein, the sensitivity for urine samples was 33.3% for DHF/DSS patients and 13.8% for DF patients, much lower than noted above for the other NS1 antigen detection tests.

The success of Chuansumrit, et al. [166] with NS1 detection in urine and the results presented in this dissertation provide proof of concept that NS1 antigen can be detected in urine specimens of DEN patients. More effort in this area could potentially increase the sensitivity of the approach and could potentially lead to development of a point of care diagnostic test using a non-invasive clinical specimen. Detection of NS1 antigen in urine is also provocative in terms of understanding the pathogenesis and tissue tropisms of DENV in humans. Research efforts to investigate the role of the kidney in DENV pathogenesis and the mechanisms by which NS1 antigen enters the urinary tract would be a fruitful area of research.

There are no previous reports on the detection of NS1 antigen in saliva of DEN patients. In this study, NS1 was detected more frequently in saliva than in urine samples (Table 6), and the mean A_{450} values were higher for saliva compared to urine samples (Tables 7 and 8). Statistical analysis indicated that serum is a better specimen to detect NS1 antigen than either urine or saliva specimens. However, the low sensitivity of the Platelia NS1 test for detection of NS1 in saliva of DEN patients (27.7%; 23 NS1 positives out of 83 tested samples) still precludes its clinical use**.** Potentially an optimization of the Platelia NS1 test for detection of DENV NS1 in saliva could increase the sensitivity of the test for use in non-invasive samples. For example, sample preparation could be optimized in this kind of specimens to enrich the target.

In summary, the results presented here suggest that the commercial Platelia™ Dengue NS1 Ag kit, which was designed for use with serum specimens, is not sensitive enough as currently used to be employed as a point of care diagnostic test with urine and saliva specimens. Nonetheless, modifications in sample preparation, to the ELISA test or in the reagents used, could result in increased sensitivity.

CHAPTER 4

METABOLOMICS-BASED DETECTION AND PROFILING OF CANDIDATE SMALL MOLECULEBIOMARKERS IN SERUM, SALIVA AND URINE ASSOCIATED WITH DENGUE AND SEVERE DENGUE INFECTION OUTCOMES

4. A. INTRODUCTION

Approximately 30% of dengue (DEN) patients will progress to severe disease, which can be fatal unless timely detected and managed.[4, 5] A critical need is the ability to predict, using acute phase clinical specimens, whether or not a patient will progress to severe DEN disease. Similarly, the ability to diagnose and predict the outcome of dengue virus (DENV) infections using non-invasive, acute phase clinical samples is of critical importance.

New approaches are clearly needed to provide diagnostic and prognostic biomarkers for DEN disease outcomes. Metabolomics provides an innovative approach to identify candidate small molecule biomarkers (SMBs, molecular features, candidate compounds, and/or identified metabolites) that are altered during DENV infection resulting from perturbation of the host metabolome. Metabolic changes can be directly attributable to infection by reflecting host responses to the virus and to tissue damage, inflammation, and other pathogen induced pathology reflected in host metabolic pathways.[213]

Metabolites provide a functional overview of cellular biochemistry in diseased and non-diseased states. Emerging metabolomic technologies allow for the detection and measurement of SMBs, which could aid in the development of a disease-specific metabolite profile.[56] A metabolomics approach could identify a biosignature of SMBs that differentiate DEN disease states and that could diagnose and prognose whether or not the patient will experience DF disease or will progress DHF/DSS

Nonetheless, it is unlikely that LC-MS/MS technology as currently constructed will be applicable in the front line clinics where DEN patients first present. An ideal test for front line clinics would provide diagnosis and prognosis of DENV infections in one platform (e.g. an ELISA based test capable of

detecting multiple analytes) that could be easily and quickly applied in clinical settings as a point-of-care (POC) diagnostic test. However, identification of new biomarkers for eventual construction of such tests is needed. Metabolomics provides an innovative approach for identifying such biomarkers. Metabolic alterations can be caused by enveloped viruses, such as DENV, which are dependent upon host cell lipid synthesis for replication in cells. Consequently the infected-cell homeostasis of metabolites related to phospholipids, sphingolipids, lipid intermediates and fatty acids is affected during infection.[8] *In vitro* studies have revealed that DENV infection alters the cell metabolome [8, 280]. Perera, et al., demonstrated that DENV infection perturbs lipid homeostasis and induced metabolic changes in mosquito cells.[8] In earlier studies, Brooks et al.[280] investigated metabolic changes in DENV infected cultured vertebrate cells by frequency-pulsed-electron-capture gas-liquid-chromatography (FPEC-GLC). Comparison of cultures infected with the four DENV serotypes revealed spectra (presence or absence of selected peak spectrum of interest) that were significantly different for each serotype.[280]

A similar study using H+NMR (proton nuclear magnetic resonance) and mass spectrometry was performed to determine the metabolic profile of cultured endothelial cells that were infected with different DENV serotypes. The differences in metabolites between serotypes included amino acids, dicarboxylic acids, fatty acids and organic acids related to the tricarboxylic-acid (TCA) cycle.[281] More recent metabolomics studies have revealed that DENV infection of humans also results in metabolic changes in lipid metabolites and disease stage-specific metabolite profiles.[7] Results confirming changes in the lipidome and metabolome of humans during DENV infections are presented in this Chapter and in Chapter 5.

The purpose of this research was to use a metabolomics approach to detect and identify a biosignature panel of candidate SMBs that differentiate DEN disease outcomes (DHF/DSS, DF or ND) using acute phase clinical specimens and that would have prognostic capability for DEN disease.

An untargeted LC-MS based metabolomics approach was used to detect molecular features and candidate compounds in acute phase serum, and linked non-invasive urine and saliva samples that differed in disease outcomes (i.e., DHF/DSS, DF, or ND). Pairwise comparisons were used to identify molecular features for differentiating disease outcomes among diagnosis groups and the respective compounds were potentially identified in *silico*, by mass, retention time, and statistical analyses. This provided considerable insight into the number and categories of compounds associated with different DEN disease outcomes. Fatty acids, amino acids, nucleosides, vitamin derivatives, phospholipids and sphingolipids were the most relevant biochemical classes of potential compounds detected in this stage of the project. The identification of actual SMB metabolites using LC-MS/MS and hypotheses concerning the roles of these in DENV pathogenesis can be found in Chapter 5.

4.B. STUDY DESIGN – APPROACH AND WORKFLOW

This chapter also includes the optimized protocols applied and data collection parameters used, including the protocol for specimen preparation before LC-MS, the parameters used for LC and for MS, the data acquisition software parameters, data processing, and statistical analysis. These protocols and analyses are critical for the technical and analytically demanding metabolomics approach to identify candidate compounds of DEN disease and outcomes.

The general approach and workflow for the metabolomics-based identification of candidate SMBs of DEN disease outcomes follows (Figure 17). Briefly, acute phase serum, urine and saliva specimens from DENV-infected and ND patients presenting at clinics in Merida, Mexico and Managua, Nicaragua were kindly provided by collaborators in the respective countries (4.B.2.). Samples were prepared, subjected to LC-MS, visualization in chromatograms and by PCA, and data analysis. Candidate compounds were then potentially identified in silico.

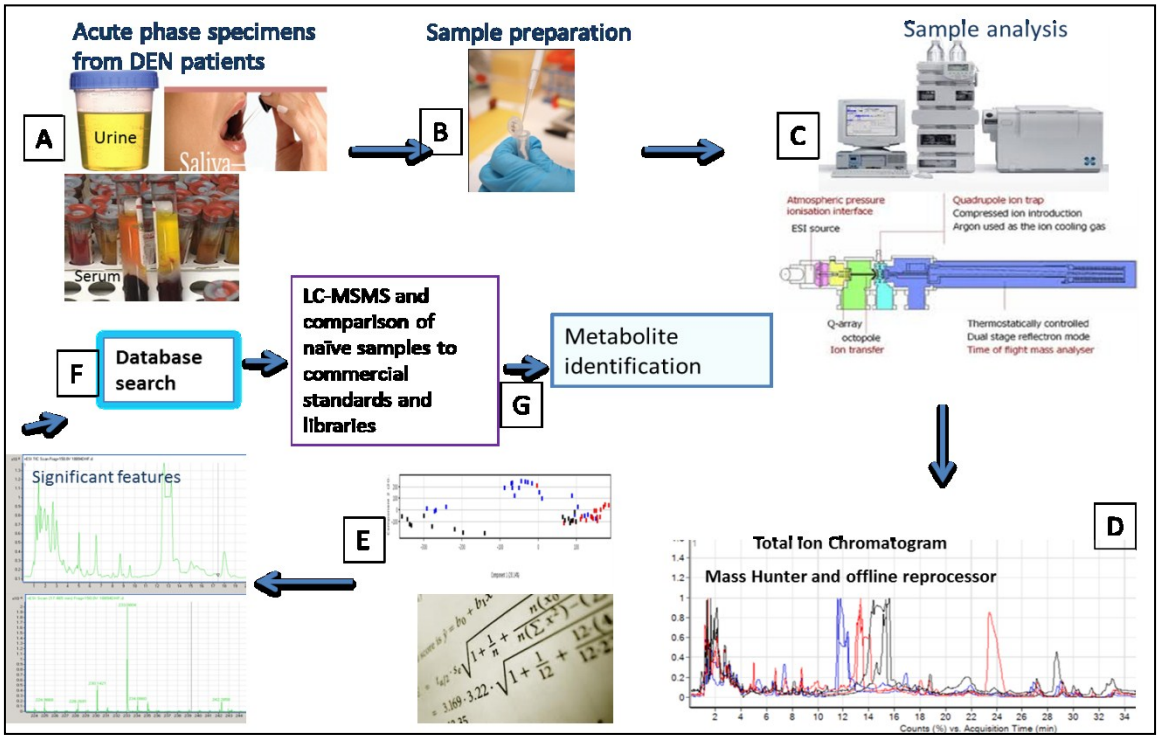


Figure 17. General metabolomics workflow and approach

Protocols for sample preparation were optimized for each type of specimen to improve the results of the mass spectrometry analysis. Prepared samples were then subjected to LC-MS using a hydrophilic interaction chromatography (HILIC) column. Thousands of features were detected with this approach (LC-MS) and were analyzed with a commercial software package (Masshunter Qualitative analysis and Mass Profiler Professional from Agilent Technologies, Palo Alto, CA) to identify candidate compounds that statistically differed in the comparison of different diagnosis groups.

The 1997 WHO Dengue guidelines were used to classify the DEN diagnosis groups, which were non-dengue (ND), dengue fever (DF), and dengue hemorrhagic syndrome/dengue shock syndrome (DHF/DSS).

The following terminology for the products of metabolism identified by LC-MS will be used in this dissertation. Molecular features, candidate compounds, and identified metabolites comprise the candidate SMBs to be identified.

- *Molecular feature* has a mass and a retention time (RT) but not a potential *in silico* identification. The small molecular features are detected in the preliminary phases of data analysis using "molecular feature-extraction (MFE)", principal components analysis, and box whisker plot. These molecular features do not have a potential identification or name; they only have a mass, RT and a calculated formula.
- *Candidate compound* is a low molecular weight biochemical compound that has been potentially identified *in silico* using the neutral mass and RT of the corresponding molecular feature. The potential identity of the candidate compounds have not been yet corroborated by LC-MS/MS.
- *Metabolite* will be used to refer to the SMB whose identity has been corroborated by LC-MS/MS analysis.

Promising differential molecular features were selected for further analysis. The selection process was based on statistically determined cut-off values (P-values $P \leq 0.05$, fold change ≥ 2, corrected P-value ≤ 0.05) that differentiated (by increased or decreased concentrations) the different diagnosis groups. The molecular features that passed the statistical cut off were subjected to a preliminary identification by investigating the neutral mass of the molecular feature in an installed library (METLIN) or in a freely available online database (METLIN and HMDB). After a preliminary identity was assigned, potential biological relevance of the *in silico* identified compounds were taken in consideration when selecting features for LC-MS/MS confirmation (Chapter 5).

4. B.1. Materials and Methods. The clinical samples and analysis procedures including protocols for sample preparation and analysis are described in detail in following sections.

4.B.2. Clinical Samples. Acute phase samples from febrile patients including serum only or linked serum, urine and saliva were initially collected from patients presenting in Mexico. Approximately three years later, similar samples were collected from patients presenting in Nicaragua. These specimens with

no patient identifiers, but with information on the final diagnosis of the case (DF, DHF/DSS, and ND), were sent to CSU for metabolomics and NS1 analyses. These studies were approved by the IRB of CSU and the responsible human investigations ethics committees at the University Autonoma of Yucatan and the "Hospital infantil Manuel Jesus Rivera", Ministry of Health, in Managua, Nicaragua.

4.B.2.a. Samples from Mexico. Specimens were collected in the Laboratory of Arbovirology of the Hideyo Noguchi Regional Research Center in Merida, Mexico. Patients who presented with a febrile DEN-like disease who were referred to the laboratory clinic by a primary-care physician had samples collected. The patients were interviewed and serum, urine and saliva specimens were collected. These samples were procured as part of the normal DEN diagnosis mission of the laboratory and not as part of an experimental protocol. Patients were from both sexes (females 53%, males 47%) and included pediatric (approximately 10% of serum samples ranged from ages 1-15 years old) and adult (ages 16-71) patients. Serum samples were collected with an aseptic technique using a sterile needle and syringe and cleaning the site of the puncture with alcohol. Aliquots of serum were made and stored in microcentrifuge tubes that were promptly frozen at -70°C. Urine was collected in sterile cups, and saliva was collected in conical tubes; both specimens were aliquoted and frozen promptly after being collected. These were then sent to CSU for metabolomics and NS1 analysis. (Table 10)

4. B.2.b. Samples from Nicaragua. In Managua, Nicaragua, serum, saliva, and urine specimens were collected as part of two ongoing studies being conducted by the University of California – Berkeley and the clinical laboratory of the Health Center Socrates Flores Vivas of the Ministry of Health of Nicaragua. The Dengue Pediatric Cohort Study, which is focused upon determining the incidence of DENV infections in children in Managua, and the Dengue Hospital study, which is focused upon identifying disease biomarkers of severe DEN infections, amended their respective protocols to collect linked serum, saliva, and urine specimens from patients presenting in either study. The linked samples were then coded and provided anonymously to CSU for metabolomics studies. Samples were collected from male and female

patients less than 15 years of age presenting in clinics or the hospital with a febrile illness. Specimens were collected and stored in a similar manner as in Mexico and sent to CSU for analysis. (Table 10)

Table 10 . Acute phase serum, urine, and saliva samples in each of the DEN diagnosis groups*

Acute Samples	SERUM			URINE			SALIVA		
	DHF/DSS	DF	ND	DHF/DSS	DF	ND	DHF/DSS	DF	ND
Mexico	26	42	33	22	32	34	15	39	30
Nicaragua	38	125	44	38	125	44	38	125	44

4.B.3. Clinical sample preparation.

4.B.3.a. Processing of serum for HILIC LC-MS analysis. Samples were thawed on ice, and an aliquot of 25 µl was placed in a new 1.7 ml Eppendorf tube. Then 75 µl (a ratio of 1:3 serum: methanol) of cold LC-MS grade methanol were added, and the vial was vortexed for 60 seconds. The sample was incubated at -20°C for 20 min to allow proteins to precipitate. The vial was centrifuged for 20 min at 14,000 rpm at 4°C. The supernatant was transferred to a new vial, and the pellet was discarded. The sample in the new vial was dried using a speed vacuum at room temperature for 45-60 min. The sample was then resuspended in the original volume (25 µl) in 100% LC-MS grade acetonitrile (ACN) solvent and incubated at room temperature for 10 min. Following vortexing for one min and centrifuging for 5 min at 4°C at 14,000 rpm, 15 µl of the supernatant were transferred to a glass vial for LC-MS analysis.

4.B.3.b. Processing of serum for reverse-phase LC-MS analysis. An aliquot of 50 µl was placed in a new vial, and 150 µl of cold methanol of LC-MS grade were added. The sample was vortexed for 1 min and incubated at -20°C for 20 min. The sample was centrifuged for 20 min at 14,000 rpm at 4°C, and the supernatant was transferred to a new vial. The pellet was discarded. The sample was dried in a speed vacuum, and resuspended in 50 µl of 5% LC-MS ACN, vortexed for 1 min, and centrifuged for 5 min at 14, 000 rpm at 4°C. 25 µl of the supernatant were then placed in a glass vial for LC-MS analysis. When the volume of sample available was less than what was needed, smaller volumes of the sample were used, but the ratio of sample: solvent was not modified.

4.B.3.c. Processing of urine samples for LC-MS. An aliquot of 50 µl was placed in a new Eppendorf tube to normalize using the creatinine concentration in urine. Urine needed to be normalized because some of the patients from whom it was collected were dehydrated. This could affect the abundance of SMBs during pairwise comparisons; thus urine specimens needed to be normalized across patients. The amount of creatinine excreted daily by an individual is relatively constant; thus urinary creatinine levels can be used as an index for standardization. Normal urinary creatinine levels for men and women are 9.7-24.7 and 7.9-14.2 mmol/24h, respectively. A "Creatinine Microplate Assay kit™" (CR01) from Oxford Biomedical Research was used to measure creatine levels in the urine samples. The kit is a colorimetric assay for the quantitative analysis of creatinine levels in urine. It is based on the principle that urinary creatinine reacts with picric acid under alkaline conditions to produce an orange color that can be quantified by absorption spectroscopy near the 500 nm wavelength. Briefly, 25 µl of each sample or provided standards were added to a 96 well plate in duplicate. 180 µl of alkaline picrate reagent was added to each well, and the plate was placed on a shaker at room temperature for 10 min. The absorbance values were determined at 490 nm. After the first reading, 15 µl of acetic acid solution (19.9%) were added to each well and mixed thoroughly in a shaker at room temperature for 5 min. The plate was read again at 490 nm. For the calculations, the absorbance values of the second reading were subtracted from the values of the first reading. The difference in absorbance between the two samples is directly proportional to the creatinine concentration. A standard curve was created with the difference of the readings in the Y axis, and the creatinine concentration of the standards in the X axis (Figure 18). An equation was calculated from the trendline in the graph, which was used to calculate the creatinine concentration in mg/dl units. Once obtained, all samples were normalized to 10 mg/dl with addition of LC-MS grade water. After the normalization, 50 µl of urine were transferred to a new vial, vortexed, and centrifuged at 14,000 rpm at 4°C for 20 min. 25 µl of the supernatant were transferred to a glass vial to be analyzed by LC-MS or LC-MS/MS.

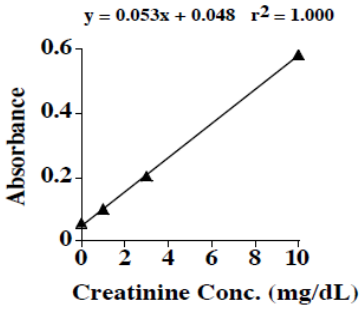


Figure 18. Example of a standard curve used to calculate the creatinine concentration in urine. Y axis represents the absorbance and X axis the creatinine concentration.

4.B.3.d. Processing of saliva samples for HILIC LC-MS. Saliva samples were allowed to thaw at room temperature. Once in liquid state, the sample was centrifuged at 14,000 rpm for 20 min at 4°C, and then 50 µl of supernatant were collected and placed in a new vial. 100 µl of ACN (LC-MS grade) was added (ratio 1:2 sample: solvent), and the vial was vortexed for 1 min and placed at -20°C for 10 min in order to precipitate proteins present in the sample. It was centrifuged at 14,000 rpm at 4°C for 5 min.[282] Then, 25 µl was transferred to a glass vial for LC-MS analysis.

4.B.4. Liquid Chromatography (LC). The data presented in this dissertation were collected using HILIC LC-MS. A subset of 46 serum samples were analyzed using RP LC-MS, but this approach was not pursued as part of this dissertation. Nonetheless the methodology used for RP and the results of LC-MS will be briefly compared with HILIC LC-MS methodology and results.

An Agilent 1200 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA) was used in the LC studies. For the analysis, the samples were dissolved in a non-polar solvent (ACN was selected as preferred solvent due to better compatibility with HILIC columns) and loaded onto the column. Non-polar analytes preferentially associated with ACN and moved through the column with little retention. Conversely, polar analytes associate with the stationary phase and are retained. The mobile phase was then gradually changed to increase water content, and polar molecular features began to elute from the column. The size of the column was 4x100mm. The

bonded phase was a diamond hydride, pore size 100 Å , surface area 30m^2/gm, pore volume 0.92 ml/min, paticle size 4µm, Carbon load <2%, pH range 2.5-7 and maximum temperature 60°C. The volume of the sample injected was 5 µl, and flow rate of the sample through the column was 0.4ml/min.

Before the start of an analysis, the column was cleaned with 50:50 MeOH/water for 45 min, then the column was conditioned by 95% acetonitrile/5% water/1% formic acid for 45 min using the gradient mentioned in Table 11, which was followed by 20 min of column re-equilibration with no gradient. The conditioning step (65 min in total) was repeated for at least 12 cycles. The column was cleaned as indicated above for 45 min after the analysis of every 15 specimens. After cleaning, the column was re-equilibrated and conditioned for 65 min as mentioned above before injecting another batch of 15 samples.

The chromatography conditions used for separation included solvent A: Water, 0.1% formic acid, Solvent B: Acetonitrile, 0.1% formic acid, and calibration reference ions: m/z 121.050873 and 922.009798. The chromatography gradient is provided in Table 11.

Table 11. Liquid-chromatography gradient*

Time	Gradient
0.2 – 30 minutes	95% B to 50% B
30 – 35 minutes	50% B
35 – 40 minutes	50% B to 20% B
40 – 45 minutes	20% B to 95% B

*Non-linear elution gradient used in liquid chromatograph. The gradient is expressed as percentage of solvents (acetonitrile (B) to water) changes at different time points during LC-MS/MS analysis. Water accounts for the missing percentage.

4.B.4.b. Mass spectrometry (MS).The eluent from HPLC was introduced into the Agilent 6520 Quadrupole time of flight (Q-TOF) mass spectrometer coupled with dual electrospray ionization (ESI) (Table 12). MS analysis was performed with the same solvents as mentioned above for LC and the same reference ions. The data were collected in positive mode in centroid and profile mode in 4 GHz high resolution. Data collection for MS lasted for 45 min.

Table 12 . Description of time of flight mass spectrometry parameter values for detection of *m/z* of molecular features from prepated samples*

MS Component	Parameter
Ionization	Dual ESI ionization source
Scan Rate	1.4 spectrum/sec
Vcap	4000 V
Drying gas (N_2)	325 °C at 10 L/min
Nebulizer pressure	45 psi
Fragmentor	150 V
Skimmer	65 V
OctopoleRFPeak	750 V
Capillary pump	Flow 40µl, pressure 400 bar
Binary pump	Flow 0.4ml/min pressure 400 bar
Mass range	100-1700 Da
Calibrated	<2 ppm mass accuracy

* Abbreviations of parameters: Vcap = capillary voltage; V=Volts; Da=Dalton; ppm=parts per million; ESI= electrospray ionization.

4.C. DESCRIPTION OF DATA ANALYSIS AND WORKFLOW. The optimized data analysis workflow consisted of the following steps:

4.C.1. Molecular feature extraction from raw data. Data from the TOF LC/MS were exported to Mass Hunter Qualitative analysis (Qual) and subjected to molecular feature extraction (MFE). MFE identified peaks in the total ion chromatogram raw data and reduced the complex data set to a simple list of calculated chemical formulas/ potential molecular features considering accurate mass, retention time, intensity and the list of ions associated with each peak. The algorithm used the parameters described in Table 13.

Table 13 . Description of parameter values used in Mass Hunter Qualitative analysis for molecualr feature extracion (MFE) from raw data*

Molecular feature extraction on raw data	
Target data type	Small molecular chromatographic
Use peaks with height	>=600 counts
Ion species allowed	+H and +Na
Charge state	isotope grouping
Peak spacing tolerance	0.0025 m/z, plus 7.0 ppm
Compound filters	Height (absolute height) >=3000 counts
Peak smoothing	0.2 times peak width
Compound ion threshold	two or more ions

*The data were exported to a file (.cef) for the next step in analysis in mass profiler professional (MPP).

4.C.2. Standardization of molecular features. The unidentified molecular features extracted in the previous step were imported into MPP; samples were grouped into the experiment parameters, which included DHF/DSS, DF and ND diagnosis groups. The molecular features were subjected to filtering, alignment, normalization and baselining with the parameters described in Table 14.

Table 14. Description of parameter values for standardization of raw data*

Parameters	Values
Filtering	
Minimum absolute abundance	5000 counts
Minimum number of ions	2
Charge states	All charge states permitted
Alignment	
RT window	0% + 0.25 min
Mass window	15 ppm + 2 mDa
Normalization	
Percentile shift	75
Baselining	
Baseline to	Median of all samples
Interpretation	
Select experiment parameters	Diagnosis groups (DHF/DSS, DF, ND)
Display mode	Categorical
Average over replicates in conditions	Non-averaged
Use measurements flagged	Present and Marginal (exclude absent)
Filtering	
Entity list	All available entities
Interpretation	Non-averaged diagnosis
Retain entities that appear in at least	50% of samples in at least one condition

*MPP was used to standardize the molecular features obtained from raw data in previous step before subjecting the features to significance analysis.

The molecular features obtained with the above parameters were preliminarily identified using the molecular formula generator (MFG). This step assigns the molecular features a calculated chemical formula and a potential identity (Table 15). The chemical formula is necessary for the next step of the

workflow. MFG is used once more in the final steps of the workflow to corroborate identities of statistically significant features.

Table 15. Molecular formula generator algorithm to assign molecular formulas and potential identities of molecular features

Compound identification wizard in MFG	
Values to match	Mass match tolerance: 5 ppm
Database	METLIN (database is installed)
Maximum number of peaks to search when peaks are not specified graphically	5
Charge carried and charge state range	H+ and 1
Scoring (contribution to overall score)	
Mass score	100
Isotope abundance score	60
Isotope spacing score	50
Retention time score	100
Expected data variation	
MS mass	2.0 mDa + 5.6 ppm
MS isotope abundance	7.5%
Generate formulas	
Positive ions	H+
MS ion electron state	Even electron
Elements and limits (>minimum # and<maximum number)	C >3 and <60 H >0 and <120 O >0 and <30 N >0 and <30 S >0 and <5 Cl >0 and <3
Maximum neutral mass for which formulas were calculated	1,000 Da
Limits on results: minimum overall score	35
Charge state, peak spacing tolerance	0.0025 m/z + 7 ppm
Isotope model	Common organic molecules
Limit assigned charge states to a maximum of	2

*MFG parameters were used to assign accurate chemical formulas and identities to molecular features. Scoring includes a minimum quality score for each type of parameter. If the quality of any compound was below the minimum quality score then it was filtered out.

4.C.3. Visualization of data by principal component analysis and box whisker plot analysis. A non-parametric, **principal component analysis (PCA)** (e.g. Figure 20) is a scatter plot of data projected along

the principal axes (X, Y and Z). PCA captures the variance of each sample in order to visualize major trends of the data and the clustering (or the absence of it) of samples by diagnosis. A non-parametric, unbiased, **box whisker plot** (e.g. Figure 21) was used to graphically depict the diagnosis groups through their quartiles and to visually assess the characteristics and quality of the data that is being analyzed.

Box whisker plots were performed by averaging all the molecular features contained in every sample in a given diagnosis group (represented by a bar in the diagram). This revealed that there was variability of abundance of molecular features among diagnosis groups and a considerable number of outliers in Nicaraguan and Mexican samples. Both figures suggested that the variability detected among diagnosis groups could be investigated to potentially differentiate the respective diagnosis groups and that the data from urine and saliva had a lower quality than data from serum specimens

The box whisker plot figure presents the dispersion or variability of the different abundances of the molecular features detected in all the samples within a diagnosis group (in terms of interquartile ranges, the variability or dispersion equals the difference between upper and lower quartiles). The lines extending vertically indicate variability outside the upper or lower quartiles. A quartile is one of the three points that divide the data into four equal groups, with each group comprising a quarter of the data. The outliers were plotted as individual red lines.

In summary, a PCA (e.g. Figure 20) represents the diagnosis data of each sample without averaging it into diagnosis groups. In the diagram, each dot represents an individual sample, while, in the opposite fashion, the box whisker plot (e.g. Figure 21) represents the averaged data by diagnosis group, shown by a rectangle representing all samples within each diagnosis group.

4.C.4. Find by Molecular formula. The standardized molecular features from the previous step in MPP (Tables 14 and 15) were imported into Masshunter Qual once again. This recursive step has the purpose of matching the formulas of standardized molecular features with the ones from the raw data. If the molecular features from the standardization step and the ones from the raw data match, these are used

in the statistical analysis, which is the next step of the workflow. The parameters used in this algorithm to find by molecular formula are included in Table 16.

Table 16 . Description of parameter values for "Find by molecular formula" algorithm*

Find compounds by formula	
Maximum number of matches allowed	One. Mass and retention time are required to match a compound.
Match tolerance for mass and retention time	+/- 10 ppm and 0.25 min
Positive ions and charge state	H+ and 1
Scoring	Same as Table 15
Result filters	
Only generate compounds for matches formulas	
Do not match if score is	<60%
Smoothing function	Gaussian
Mass spectrum	
Average scans	10% of peak height
TOF spectrum, exclude if above	10% of saturation
Peak spectrum background	Average of spectrum at peak start and end
Peak location	Maximum spike width=2
Required valley	0.7
Charge state	
Same as Table 15	

*Algorithm for recursive analysis to confirm that the molecular features are in fact present in the raw data. The results were saved and exported to a (.cef) file for the next step in the analysis.

4.C.5. Significance analysis. Once the presence of molecular features were corroborated, statistical analysis was performed to determine if there were differences in abundance of the individual molecular features that differentiated the diagnosis groups. The significance analysis workflow was as follows. A non-averaged interpretation was used for statistical analyses. In an *averaged* interpretation, the mean intensity value for each molecular feature across the replicates is used for analysis and visualization. In a *non-averaged* interpretation, the normalized intensity value for each molecular feature in each sample is used for analysis and visualization. Non-averaged interpretations are useful for assessing sample variance using PCAs and cluster analyses. The non-averaged interpretation uses the abundance of the molecular feature without standardizing it to every sample included in the diagnosis group; the intensity

of each molecular feature in each sample is analyzed separately, which allows for analysis using the diagnosis group as the variable of interest. The software will use averaged values for statistical analysis regardless of the type of interpretation selected.

The steps mentioned in Table 14 for data standardization were performed using the molecular features obtained from the previous step. The data was then analyzed to determine the statistical differences in the abundance of each molecular feature in pairwise comparisons of the diagnosis groups.

The values that represent the changes of abundance of each molecular feature among diagnosis groups (DHF/DSS vs. DF, DHF/DSS vs. ND, and DF vs. ND) were obtained with Volcano Plots using T test and fold changes for the urine and saliva specimens and ANOVA and fold change for serum specimens.

For all kinds of specimens, a false discovery rate test was determined, using the Benjamini-Hochberg algorithm with a corrected P value cut-off ≤ 0.05.

The molecular features that yielded a P value and corrected P value of ≤ 0.05 and a fold change ≥ 2 (for non-invasive samples FC ≥ 1) were imported into the MPP algorithm "molecular formula generator" (MFG) (Table 15) as part of the recursive analysis for identification. Those molecular features that were statistically significant by differences between diagnosis groups were subjected to identification by using the installed METLIN database.

For a typical serum analysis, the MFE features were compared statistically for their presence and abundance in the different diagnosis groups. This statistical analysis reduced the number of molecular features that differentiated the DEN diagnosis groups considerably and permitted focusing of LC-MS/MS identification on those molecular features that were statistically relevant.

4. D. RESULTS.

The results presented include serum analyzed by HILIC and RP, and urine and saliva specimens analysis by HILIC LC-MS. Briefly, visual tools used during the analysis such as TIC, PCAs and whisker plot are

presented, followed by figures illustrating the FC of the compounds and tables including the statistical values of the selected compounds.

4. D.1. LC-MS Total Ion Chromatogram. The total ion chromatogram (TICs) are visual tools that are used to evaluate the quality of the chromatography and to determine if there are shifts or drifts from chromatogram to chromatogram caused by chromatography differences that need to be addressed and corrected during data analysis. Examples of TICs resulting from LC-MS analysis of Mexican serum, urine and saliva specimens color-coded by diagnosis groups are presented in Figure 19.

The visualization of the raw serum data by TIC (top chromatogram) showed one large peak and a few small ones at min 1-3 and three more peaks at 14-18 and 30 min. Although only a few individual peaks are visible, thousands of molecular features are present. The urine TICs (middle chromatogram) provided a preliminary overview of sample variability, represented by peaks appearing from 1-34 min. This variability was found commonly in urine samples. Saliva TICs (bottom chromatogram) also revealed considerable variability in molecular features. The variability in urine and saliva samples may be attributable to differences in age, diet, and the hydration state at the time t of sample collection. The TICs also provide an overview of molecular feature differences among the diagnosis groups as shown by color coding. Differences in peaks suggest perturbation of the metabolome by DENV infection or lack thereof in the different diagnosis groups.

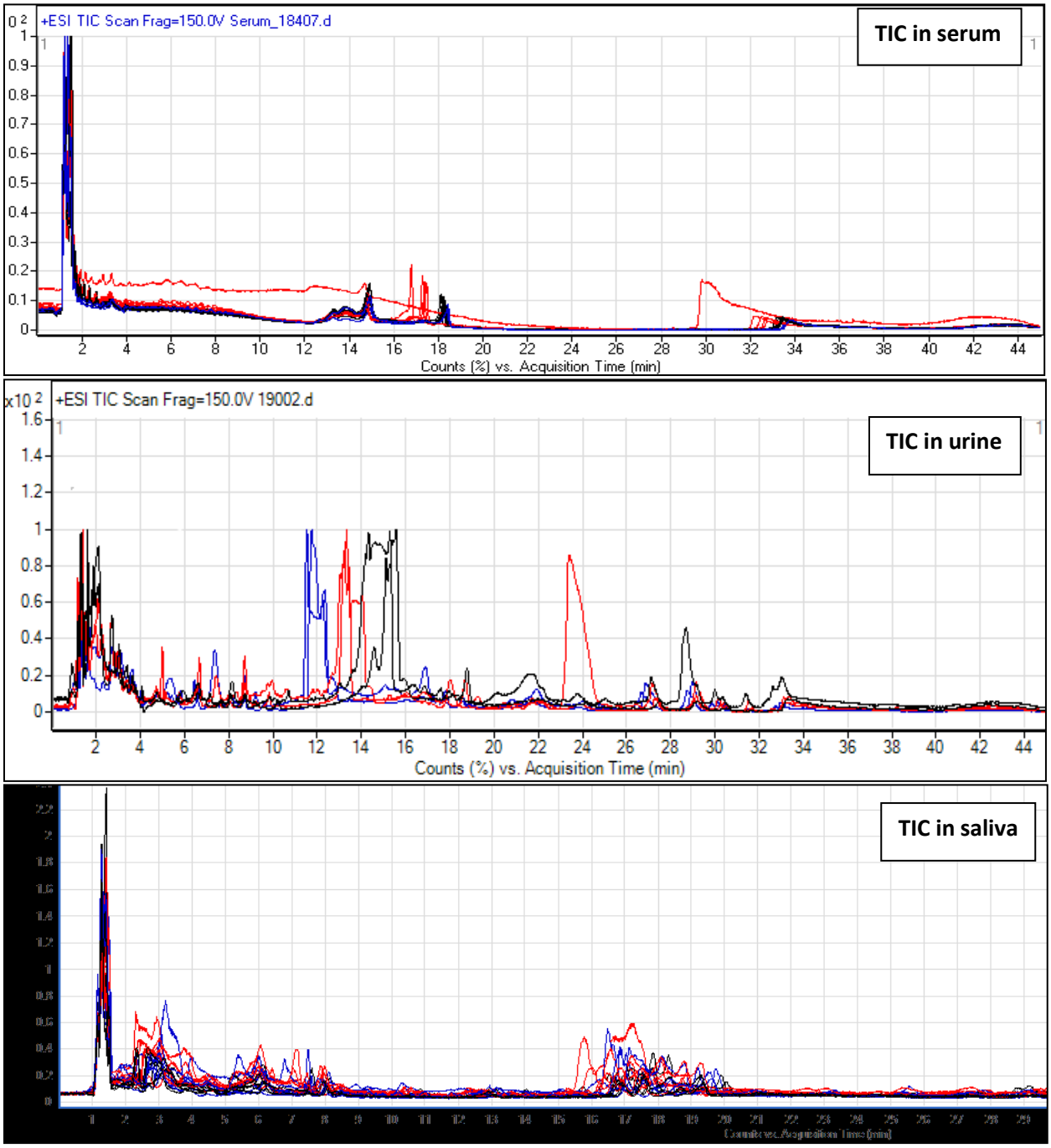


Figure 19. Total Ion Chromatogram of selected specimens. Example of superimposed TICs of selected chromatograms of Mexican serum, urine and saliva respectively. Y axis - intensity counts, X axis - retention time. Color coding: red – DHF/DSS; blue-DF, and black-ND.

4. D.1.b. Principal component analyses of molecular features. Following LC-MS, molecular features detected in Nicaraguan and Mexican serum, urine and saliva samples were subjected to PCA analysis**;** 3-D graphs were chosen to represent the results (Figure 20). Each dot in the three-dimensional (3-D) figure represents an individual sample. The size of the circle in this PCA is standard and is the same for each sample. Because it is a 3D graph, the dots appear to be larger or smaller as a matter of perspective based on any given axis being closer to the reader. The sizes of the dots can change when the graph is oriented in a different 3-D direction. In the PCAs of Nicaraguan urine and saliva, the axis is inverted (relative to serum PCAs), to provide a better view of the patterns present in the figure. The inverted axis does not affect the statistical analysis results. PCA analyses of molecular features in Mexican and Nicaraguan samples revealed that there are differences in molecular feature composition in the samples from the different diagnosis groups, which resulted in visible clustering of samples or patterns from different groups in the analysis. (Figure 20)

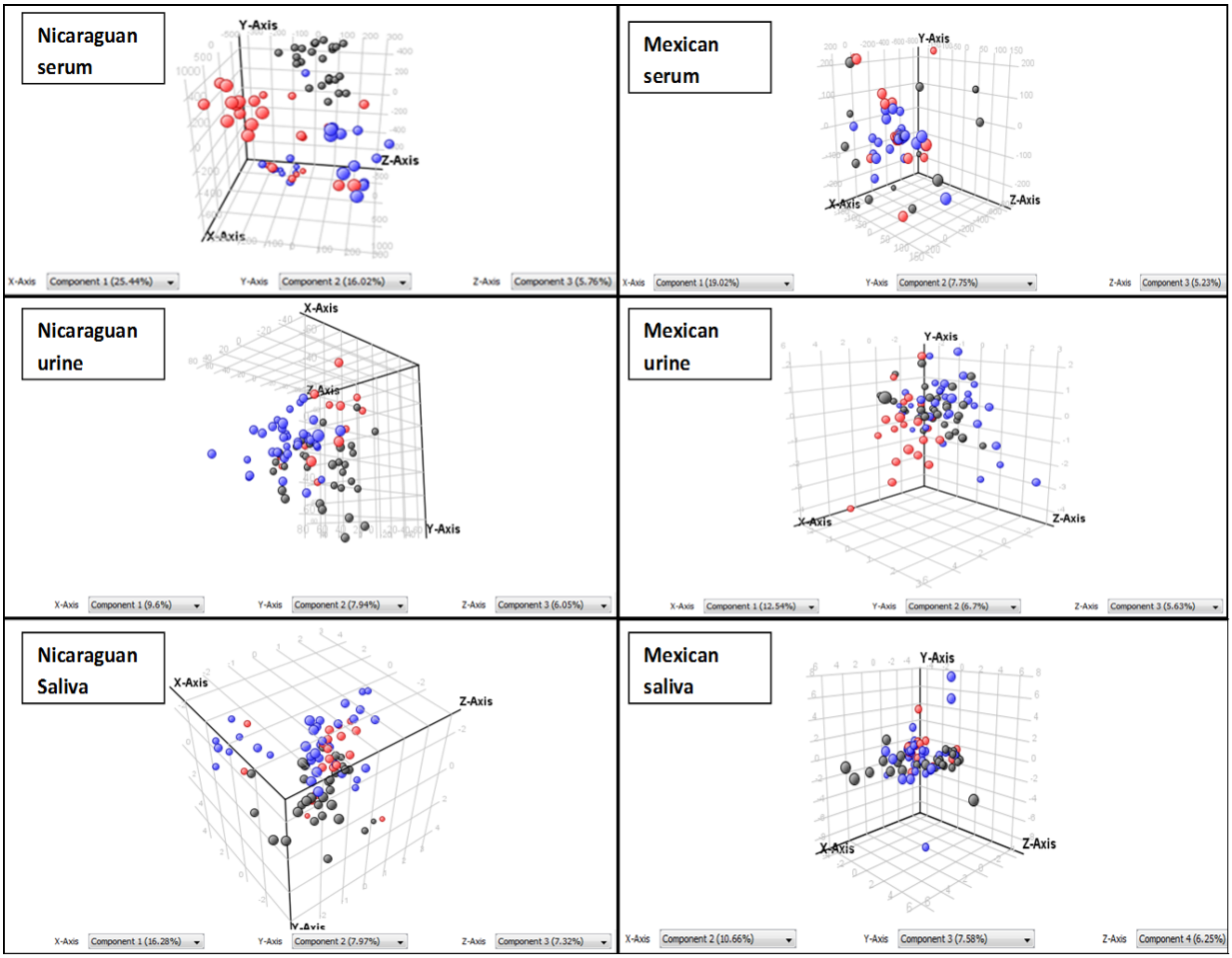


Figure 20. Principal component analysis of Nicaraguan and Mexican specimens. PCAs of serum (top), urine (middle), saliva (bottom) metabolite profiles from patients with different outcomes of DEN disease. Nicaraguan samples (left) and Mexican samples (right). Red spheres are patients with DHF/DSS, blue spheres are patients with DF, black spheres are patients not infected with DENV (ND).

Mexican diagnosis groups (Figure 20). In the PCA of Nicaraguan serum samples, there was some overlap of seven DHF/DSS samples with the DF cluster samples, but no DF samples were found in the main DHF/DSS cluster. In addition, the ND cluster was very well defined in Nicaraguan serum specimens. In the PCA of Mexican serum samples (Figure 20), the ND samples were located in the periphery of the figure and did not cluster. These control samples were obtained from febrile patients who were diagnosed as ND. The DF and DHF/DSS patients formed a loose cluster in the center of the PCA figure.

PCAs of urine specimens did not reveal clear segregation by diagnosis group, which could be due to urine variability and the variability in the abundances of the molecular features contained in each sample. The axis was turned (relative to serum PCAs) in the Nicaraguan group to provide a better view of the patterns present in the figure. The DF and DHF/DSS groups formed a large cluster predominantly at the left of the figure; the ND patients clustered in the lower right portion of the figure (Figure 20). The Mexican urine samples did not clearly segregate by diagnosis group. However, a pattern can be noticed in which the DHF/DSS samples were predominantly on the left of the figure, ND samples in the middle, and DF samples in the right (Figure 20).

PCAs of saliva specimens did not reveal segregation of patients by diagnosis group (Figure 20) In the Nicaraguan group, there was a general pattern of DF samples being on top of the figure, DHF/DSS samples in the middle, and ND samples in the bottom. However there was no discernible segregation of specimens by diagnosis group. PCA of the Mexican saliva samples also revealed that samples from the three diagnosis groups did not segregate. There was no discernible clustering of samples, making it difficult to discern a pattern or differentiation among the diagnosis groups.

In summary: PCA analyses performed with Mexican and Nicaraguan serum specimens suggested variation in molecular features contained in clinical specimens in different diagnosis groups. PCA analysis of urine and saliva specimens did not show a specific clustering of diagnosis groups, but in some instances, patterns were seen that indicate trends among diagnosis groups. PCA provided proof of principle that there are differences in molecular features in the acute phase serum clinical samples of the DHF/DSS, DF and ND diagnosis groups.

4. D.1.c. Box whisker plots analyses of Nicaraguan and Mexican specimens. The box whisker plot analyses were conducted to assess the quality of the data found in the analyzed specimens. The box whisker plots of the analyzed specimens presented in the figures (Figure 21) illustrate the variability of

molecular features among diagnosis groups and the abundance of all the molecular features included in the samples by an averaged interpretation based on diagnosis groups.

Nicaraguan serum specimens showed less variability, while urine and saliva specimens showed several outliers (molecular features with higher or lower abundance than the mean abundance of the averaged molecular features in each diagnosis group). Overall, the box whisker plot analyses suggested that the molecular feature variability detected among diagnosis groups could be exploited to differentiate the disease groups. The analyses also suggested that the data from the urine and saliva LC-MS analyses was of lower quality that the data from the serum analyses. The reasons for this remain to be determined.

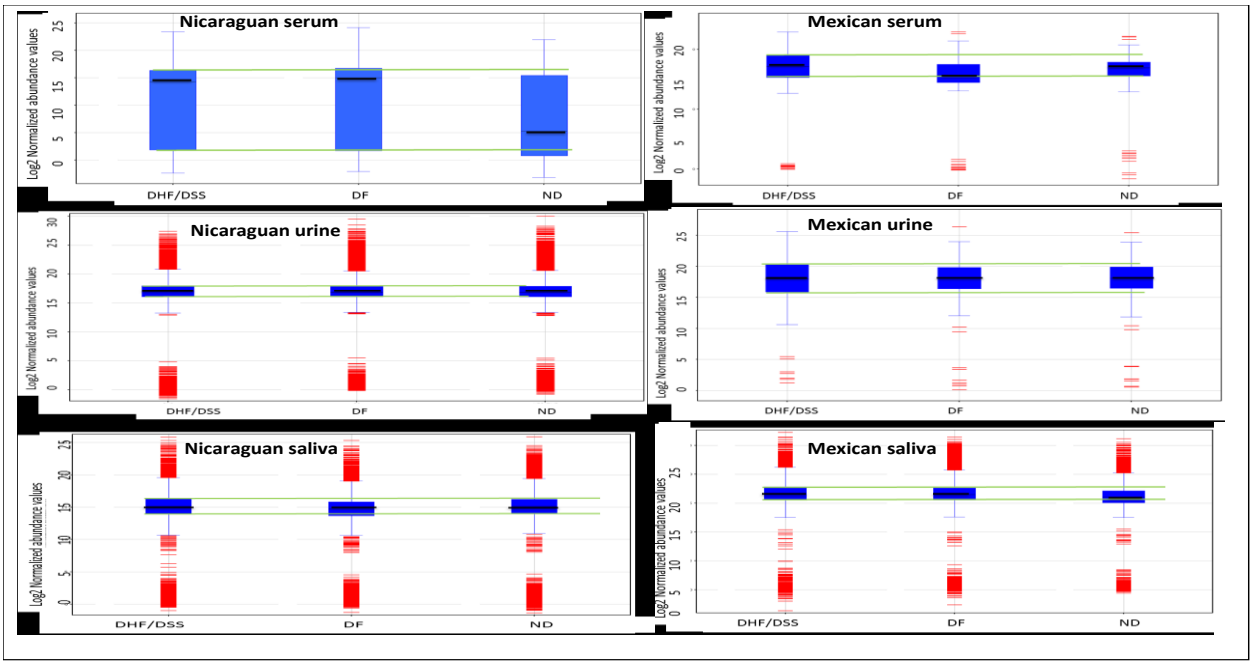


Figure 21. Box whisker plot of Nicaraguan and Mexican samples. Results are presented by diagnosis group and be clinical specimen type (serum (top), urine (middle) and saliva (bottom) figures. Columns are DHF/DSS,DF, and ND, respectively. Left figures - Nicaraguan samples; right figures - Mexican samples. Green lines are for better visualization of the variability in the abundance of molecular features within and between diagnosis groups. Mean of each group is shown in each of the rectangle by the black line.

4. D.2. Metabolomic analysis of acute phase specimens from the DF, DHF/DSS, and ND diagnosis groups. The molecular feature extraction algorithm detected approximately 2,000 features that were

extracted from serum raw data files; approximately 4,000 molecular features were detected and extracted from raw data files of urine specimens, and approximately 8,000 molecular features were extracted from raw-data of saliva samples. Molecular features detected by HILIC LC-MS in serum specimens of patients from the different diagnosis groups were subjected to statistical analysis. P-values were calculated based on the differential abundances of the molecular feature. The fold change (FC) reflected the ratio of normalized differences in the intensities of the respective molecular features between pairs of different diagnosis groups. If the molecular feature was not detected in a sample, it was excluded.

The numbers of molecular features detected in serum that differentiated the diagnosis groups are presented in Tables 17. (HILIC LC-MS) and 18. (RP LC-MS). HILIC LC-MS analysis identified 85 features in Nicaraguan serum specimens and 36 in Mexican serum specimens that differentiated DHF/DSS and DF patients (Table 17.). In contrast only 77 molecular features were detected by RP LC-MS (Table 18).

The lists of total molecular features identified by HILIC LC-MS that statistically differentiated the different diagnosis groups in serum are presented in Appendices 1 and 2. The list of total molecular features identified by RP LC-MS that differentiated the respective diagnosis groups is presented in Appendix 3.

Table 17. Total number of molecular features detected in serum by HILIC LC-MS that differentiate the DEN diagnosis groups*

Serum	DHF/DSS-DF	DHF/DSS-ND	DF/ND
Nicaragua	85	211	208
Mexico	36	325	326

*Results based upon pairwise comparisons of molecular features using cut off values of P-value of $\leq$ 0.05 and a FC of $\geq$2, corrected P-value of $\leq$0.05.

Table 18 . Total number of molecular features detected in serum by RP that differentiate the DEN diagnosis groups*

Serum	DHF/DSS-DF	DHF/DSS-ND	DF/ND
Mexico	77	90	31

* Results based upon pairwise comparisons of molecular features using cut off values of P-value of ≤ 0.05 and a FC of ≥2, corrected P-value of ≤0.15.

The numbers of molecular features detected in acute phase urine specimens that differentiated the different disease groups are provided in Table 19. In Nicaraguan samples, 105 molecular features differentiated DHF/DSS from DF patients (Table 19). Interestingly, the number of features that differentiated these two disease groups in Mexican samples was much lower (Table 19) as were the numbers of features that differentiated DHF/DSS from ND patients and DF from ND patients. The reasons for this remain to be determined. The lists of total molecular features that statistically differentiate the respective diagnosis groups are provided in Appendices 4 and 5.

Table 19 . Total number of molecular features detected in urine by HILIC LC-MS that differentiate the DEN diagnosis groups*

Urine	DHF/DSS-DF	DHF/DSS-ND	DF-ND
Nicaragua	105	144	66
Mexico	21	22	25

*Results based upon pairwise comparisons of molecular features using cut off values of P-value ≤ 0.05 and a FC of ≥1, and corrected P-value of ≤ 0.05.

The numbers of molecular features detected in acute phase saliva samples that differentiated the different disease groups and saliva are provide in Table 20. The lists of total molecular features that differentiate the respective diagnosis groups are provided in Appendices 6 and 7.

Table 20 . Total number of molecular features detected in saliva by HILC LC-MS that differentiate the DEN diagnosis groups*

Saliva	DHF/DSS-DF	DHF/DSS-ND	DF/ND
Nicaragua	52	73	74
Mexico	18	106	100

* Results based upon pairwise comparisons of molecular features using cut off values of P-value of ≤ 0.05 and a FC of ≥1 in saliva specimens from Nicaragua and Mexico.

4.D.3. Preliminary *in silico* identification of candidate compounds and literature review to determine their potential biological significance. All the selected molecular features that differentiated the respective diagnosis groups listed in Tables 17, 18, 19 and 20 were subjected to identification by interrogating the neutral mass of each molecular feature in an online database (METLIN or HMDB) or by identification based on interrogating the installed software database in MPP (Table 15).

The *in silico* identified candidate compounds were then subjected to a brief literature review using PubMed or HMDB in order to verify the biochemical nature of the compounds and to verify that the potential compound could have a potential biological function or was associated with a metabolic pathway in humans that could be perturbed by DENV infection or by the immune response of the host, and thus could condition the viral pathogenesis and disease outcomes. This step reduced the list of potential compounds by eliminating compounds annotated in the database as synthetic compounds such as drugs, and compounds of likely bacterial, fungal, or plant origin.

The putative identification of the compounds by METLIN or HMDB and determination if the compounds were of biological origin and could be potentially relevant to DEN disease, were selection of the compounds presented in Tables 21 - 27.

It is critical to acknowledge that the compounds that were not selected for the profiling analysis but that were statistically significant in pairwise comparisons (Table 17-20) could be further analyzed and identified and could be integrated into a panel of diagnostic biomarkers.

During the putative identification of the compounds using METLIN or HMDB, often a list of more than one compound that could be selected as the identity for a particular molecular feature was obtained; this list of candidates includes parts per million (ppm) error for each compound identity. To select a candidate identity for the statistically significant molecular feature (in case there was more than one hit in the database corresponding to the searched neutral mass), two factors were considered. The first factor was the ppm difference between the introduced neutral mass and the candidate results.

Ideally the ppm closest to 0 is the most accurate. The other important consideration when selecting candidate identities for the molecular features was the biological relevance of the potential identities as mentioned above. An example is offered for clarity. In the case of the neutral mass 115.0635, the search in METLIN yielded six hits, all of which have exactly one ppm difference from the original mass searched. The candidate identifications included: L-Proline, D-Proline, pterolactam, 4-amino-2-methylenebutanoic acid, 3-acetamidopropanal, and 1-aminocyclobutane carboxylic acid. In this case a search of each candidate indicated that, briefly L-proline is synthesized by humans, D-proline is not commonly found in humans, pterolactam is a synthetic drug, acetamidopropanal is associated with the metabolism of arginine, proline, glutamate, aspartate and asparagine. The last hit was 1-aminocyclobutane carboxylic acid, which is a synthetic compound used in the development of peptide-based drug candidates. In this example, L-proline was selected as the identity that has potential biological relevance due to a previous report of this amino acid being elevated under oxidative stress in endothelial cells.[283] This selected identity was arbitrarily assigned as a “candidate” or “potential” identification, which would be confirmed or discarded by LC-MS/MS analysis.

As mentioned above, the putative identification for each molecular feature in serum, urine, and saliva specimens was obtained by searching the neutral mass in freely available online databases. It is important to note that most of the identities of the compounds found in Tables 21 - 28 have not been confirmed by MS/MS, and therefore the assigned identities should be considered putative.

4.D.3.a. Results with Nicaraguan serum specimens. In Nicaraguan serum specimens, a list of twenty compounds that differed statistically in the pairwise comparison of DEN diagnosis group was selected from the molecular features listed in Table 17 based on their biological significance and their potential role in DEN pathogenesis, as well as their satisfactory statistical values in pairwise comparisons that differentiated the disease diagnosis groups. These compounds are presented in Figure 22, which represents the positive or negative FC of each compound in pairwise comparisons of DEN diagnosis.

Thirteen compounds differentiated DHF/DSS and DF patients, twenty differentiated DHF/DSS and ND patients, and twelve differentiated DF and ND patients. Seven biochemical classes of compounds resulted from the analysis of the Nicaraguan serum from METLIN and the HMDB (Table 21): amino acids, fatty acids, eicosanoids, phospholipids, sphingolipids, vitamin D derivatives and compounds of unknown classification.

The selected candidate compounds detected in Nicaraguan serum specimens following HILIC LC-MS analysis that differentiate the DHF/DSS, DF and ND diagnostic groups are listed in Table 21 with their respective mass, RT, calculated formula, a potential identity, the percentage of samples in a given diagnosis group on which it was detected in the LC-MS analysis, the number of hits on METLIN database obtained during the compound search, and statistical values corresponding to pairwise comparisons.

Pairwise comparisons of compounds that differentiated the DHF/DSS and DF diagnosis groups yielded P-values that ranged from 2.04E-03 and a FC of -6.91 (a potential ceramide compound) that was decreased in abundance in DHF/DSS patients to 1.20E-06 with a FC of 10.94 for a fatty acid compound potentially identified as α-linoleic acid that was increased in DHF/DSS patients. (Figure 22, Table 21)

The pairwise comparison of compounds that differentiated of the DHF/DSS from ND diagnosis groups in Nicaraguan samples yielded P-values that ranged from 1.15E-09 and a FC of -12.98 for the compound identified as a phosphatidylserine isotype, which had a negative FC in DHF/DSS patients to unidentified molecular feature with a P-value of 1.27E-09 with a FC of 16.20 for an unidentified molecular feature that had a positive FC in DHF/DSS patients. (Figure 22, Table 21)

The pairwise comparisons of compounds that differentiated DF from ND diagnosis groups yielded P-values that ranged from 3.96E-10 with a FC of -13.45 for a compound potentially identified as a phosphatidylserine isotype, which had a negative FC in DF patients, to 1.98E-06 with a FC of 10.07 for a compound potentially identified as a lysophosphatidylinositol isotype that was increased in DF patients (Figure 22, Table 21).

	Proline	3,6-octadecadienoic acid	α-linolenic acid	Docosahexaenoic acid	5,6-dehydroarachidonic acid	N-eicosatetraenoyl-dopamine	LysoPS(22:1/0:0)	PS(20:2/22:4)	PS(20:2/22:4)	LysoPI(21:0/0:0)	LysoPC(16:0/0:0)	LysoPC(18:1/0:0)	PC(O-16:0/O-2:0)	Ceramide	3-Deoxyvitamin D3	25-hydroxyvitamin D3	25-dihydroxyvitamin D3	Unknown	Unknown	Unknown
DHF/DF	-2.15	8.38	10.94	7.44	6.49	1.65	-4.47	0.47	-0.03	-0.58	7.55	9.16	5.88	-6.91	8.37	4.27	-5.03	1.32	1.45	4.22
DHF/ND	-8.88	8.06	10.01	7.61	4.98	8.49	-12.28	-12.98	-9.82	9.49	7.95	9.5	5.05	0.77	9.33	-3.76	-4.96	10.95	9.31	16.2
DF/ND	-6.74	0	-0.94	0.17	-1.52	6.84	-7.81	-13.45	-9.79	10.07	0.4	0.34	-0.83	7.68	0.96	-8.03	0.07	9.64	7.86	11.98

Figure 22. Selected candidate compounds detected in Nicaraguan serum specimens by HILIC LC-MS analysis that differentiate the DHF/DSS, DF, and ND diagnostic groups. Figure presents selected candidate compounds and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents a pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

Table 21 . Selected candiate compounds detected in Nicaraguan serum specimens by HILIC LC-MS analysis that differentiate the DHF/DSS, DF and ND diagnostic groups*

Mass	RT	Potential compound	Calculated formula	# DB hits	% Detected in samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
					DHF	DF	ND	P value	FC	P value	FC	P value	FC
Amino acids													
115.0635	16.08	Proline	C5H9NO2	7	7	57	21	-	-2.15	6.65E-05	-8.88	5.37E-03	-6.74
Fatty acids													
276.2087	1.11	3,6-octadecadienoic acid	C18H28O2	118	50	4	4	2.02E-05	8.38	2.93E-05	8.06	_	
278.2245	1.1	α-linolenic acid	C18H30O2	82	58	0	8	1.20E-06	10.94	7.82E-06	10.01	_	
328.238	1.09	Docosahexaenoic acid	C22H32O2	18	50	8	4	1.98E-04	7.44	8.39E-05	7.61	_	
Eicosanoids													
302.2243	1.13	5,6-dehydro arachidonic acid	C20H30O2	66	73	38	46	7.49E-03	6.49	4.18E-02	4.98	_	-1.52
455.3088	2.36	Hydroxy-eicosatetraenoyl-dopamine	C28H41NO4	2	65	42	0	--	1.65	3.27E-07	8.49	9.68E-05	6.84
Phospholipids													
579.3536	13.23	LysoPS(22:1/0:0)	C28H54NO9P	1	15	42	92	1.88E-02	-4.47	4.19E-12	-12.28	4.54E-05	-7.81
819.5418	11.83	PS(20:2/22:4)	C46H78NO9P	1	8	4	77	_	0.47	1.15E-09	-12.98	3.96E-10	-13.45
863.5598	11.81	PS(20:2/22:4)	C48H82NO10P	8	0	0	58	_	-0.03	4.30E-07	-9.82	1.17E-06	-9.79
642.3612	1.11	LysoPI(21:0/0:0)	C30H59O12P	1	58	54	0	_	-0.58	6.21E-07	9.49	1.98E-06	10.07
495.3329	13.67	LysoPC(16:0/0:0)	C24H50NO7P	11	65	27	27	9.29E-03	7.55	4.97E-03	7.95	_	0.40
521.348	13.74	LysoPC(18:1(9Z)/0:0)	C26H52NO7P	19	65	15	15	1.02E-03	9.16	4.03E-04	9.50	_	0.34
509.3834	14.80	PC(O-16:0/O-2:0)	C26H56NO6P	12	62	29	35	9.46E-03	5.88	2.45E-02	5.05	_	_
Sphingolipids													
647.6205	1.71	Ceramide	C42H81NO3	3	46	88	42	2.04E-03	-6.91	0.75	0.77	5.61E-04	7.68
Vitamin D derivatives													
368.3435	1.66	3-Deoxyvitamin D3	C27H44	2	69	27	23	2.89E-03	8.37	6.98E-04	9.33	0.7	0.96
400.3312	1.25	25-hydroxyvitamin D3	C27H44O2	54	65	96	42	6.90E-02	4.27	0.062994	-3.76	9.91E-05	-8.03
416.3282	1.45	25-dihydroxyvitamin D3	C27H44O3	70	70	88	96	5.33E-03	-5.03	0.007352	-4.96	--	0.07
Unknown													
295.2869	1.42	Unknown	C19 H37 N O	0	65	54	0	--	1.32	1.29E-08	10.95	1.88E-06	9.64
343.22	4.5	Unknown	C11 H25 N11 O2	3	85	75	12	--	1.45	1.07E-05	9.31	5.23E-04	7.86
113.0842	1.92	Unknown	C6 H11 NO	5	80	66	15	_	4.22	1.27E-09	16.20	1.19E-05	11.98

* The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database during the *in silico* identification. The percentage of detection in each diagnosis group is a proportion of samples in which the compound was detected. The statistical differences determined by pairwise comparisons among diagnosis groups are provided. Missing values were not statistically significant. Abbreviations used are: RT-retention time, FC-fold change, DB-METLIN database, PS-phosphoserine, PI- phosphoinositol, PC-phosphatidylcholine, DHF-dengue hemorrhagic fever, DF-dengue fever, ND-non-dengue.

4.D.3.b. Results with Mexican serum specimens. In Mexican serum specimens, a list of twenty compounds that differed statistically in the pairwise comparison of DEN diagnosis group was selected from the molecular features listed in Table 17 based on their biological significance and their potential role in DEN pathogenesis, as well as their satisfactory statistical values in pairwise comparisons that differentiated the disease diagnosis groups. These compounds are presented in Figure 23, which represents the positive or negative FC of each compound in pairwise comparisons of DEN diagnosis.

From the Mexican serum analysis with HILIC LC-MS, five biochemical groups were detected and these included: eicosanoids, phospholipids, sphingolipids, vitamin D derivatives and unknowns. From these, twenty compounds were selected from the molecular features listed in Table 17 based on their biological significance in DEN pathogenesis and satisfactory statistical values in pairwise comparisons that differentiated the disease diagnosis groups; ten compounds differentiated DHF/DSS and DF patients, twenty differentiated DHF/DSS and ND patients, and twenty-one differentiated DF and ND patients (Figure 23, Table 22).

Pairwise comparisons of compounds that differentiated the DHF/DSS and DF diagnosis groups yielded P-values that ranged from 7.77E-03 with a FC of -4.74 for an unidentified molecular feature to 7.06E-03 with a FC of 6.82 for a prostaglandin isotype (Table 22).

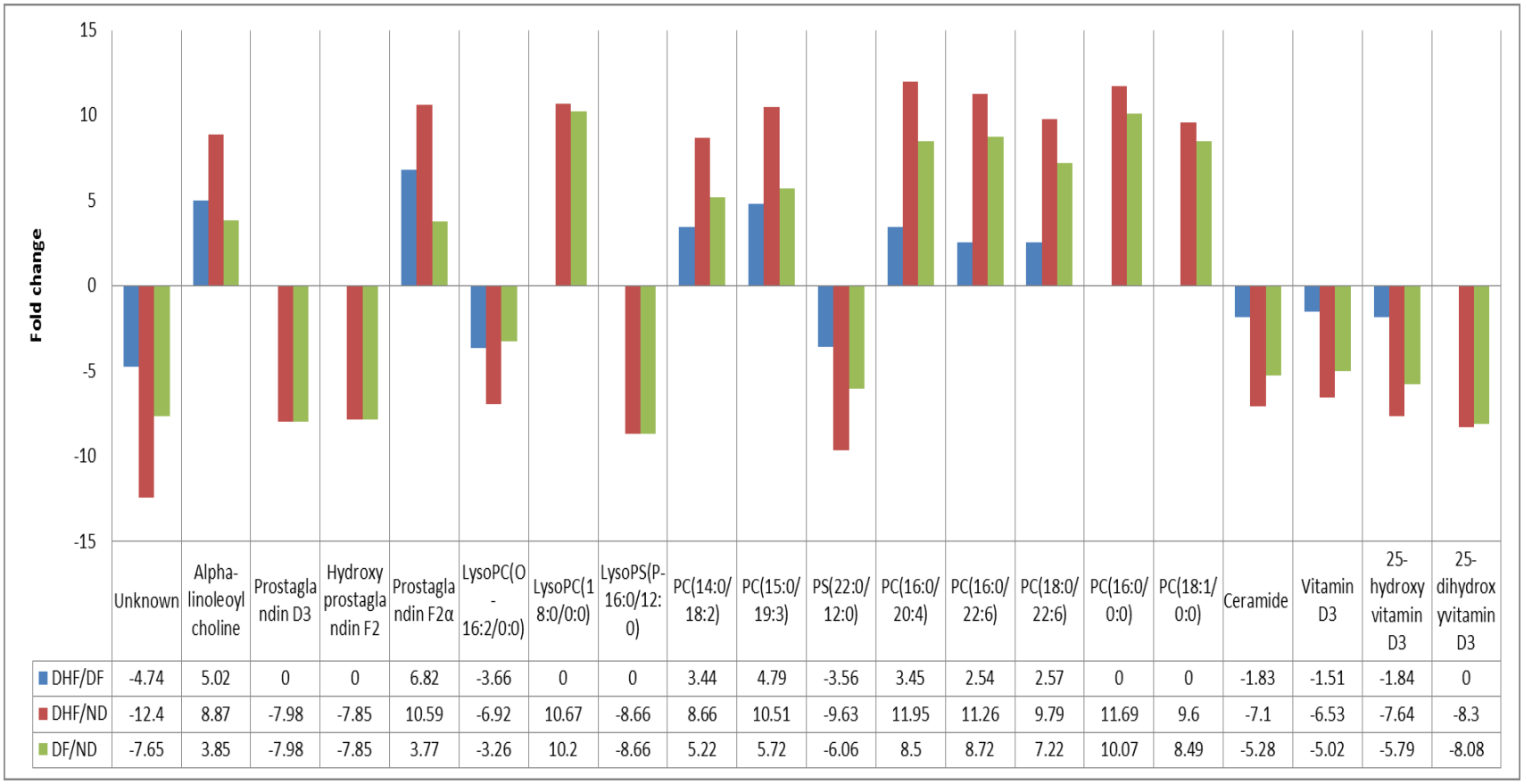
The pairwise comparison of compounds that differentiated the DHF/DSS from ND diagnosis groups yielded P-values that ranged from 6.81E-05 with a FC of -9.63 for a compound potentially identified as phosphatidylcholine isotype to a P-value of 7.21E-05 with a FC of 10.59 for a compound potentially identified as a prostaglandin isotype (Table 22). The potential prostaglandin isotype (same as DHF/DSS and DF comparison in paragraph above) could be a valuable biomarker for further evaluation and identification.

The pairwise comparisons of compounds that differentiated DF from ND diagnosis groups yielded P-values that ranged from a 3.64E-08 with a FC of -7.65 of a molecular feature not yet identified

to a P-value of 2.38E-04 with a FC of 8.50 of a phosphatidylcholine isotype (Table 22, Figure 22). The unidentified molecular feature with neutral mass of 226.1682 was kept in the list regardless of not having been identified *in silico*; the identity of this molecular feature could be annotated when databases are updated.

Reverse phase analysis yielded four biochemical categories of compounds that differentiated the DHF/DSS, DF and ND diagnosis groups, included amino acids and peptide derivatives, eicosanoids, phospholipids and unknowns. These can be found in Figure 24 and Table 23.

These biochemical groups identified by RP LC-MS analysis coincided with those identified using HILIC LC-MS. This suggests that results from HILIC and RP LC-MS analyses, which are two different analysis platforms, support and complement each other. One potential candidate compound that differentiated DHF/DSS from DF cases was detected by both RP and HILIC LC-MS analysis of Mexican sera. That candidate compound is potentially prostaglandin F2α-11-acetate methyl ester with a mass of 410.2585 with a RT of 1.36 min and 10.68 min in HILIC and RP LC-MS analyses respectively. This compound was shown to have a positive FC in DHF/DSS to DF and DHF/DSS to ND in sera from Mexican patients by both analyses (Figure 23 and 24).



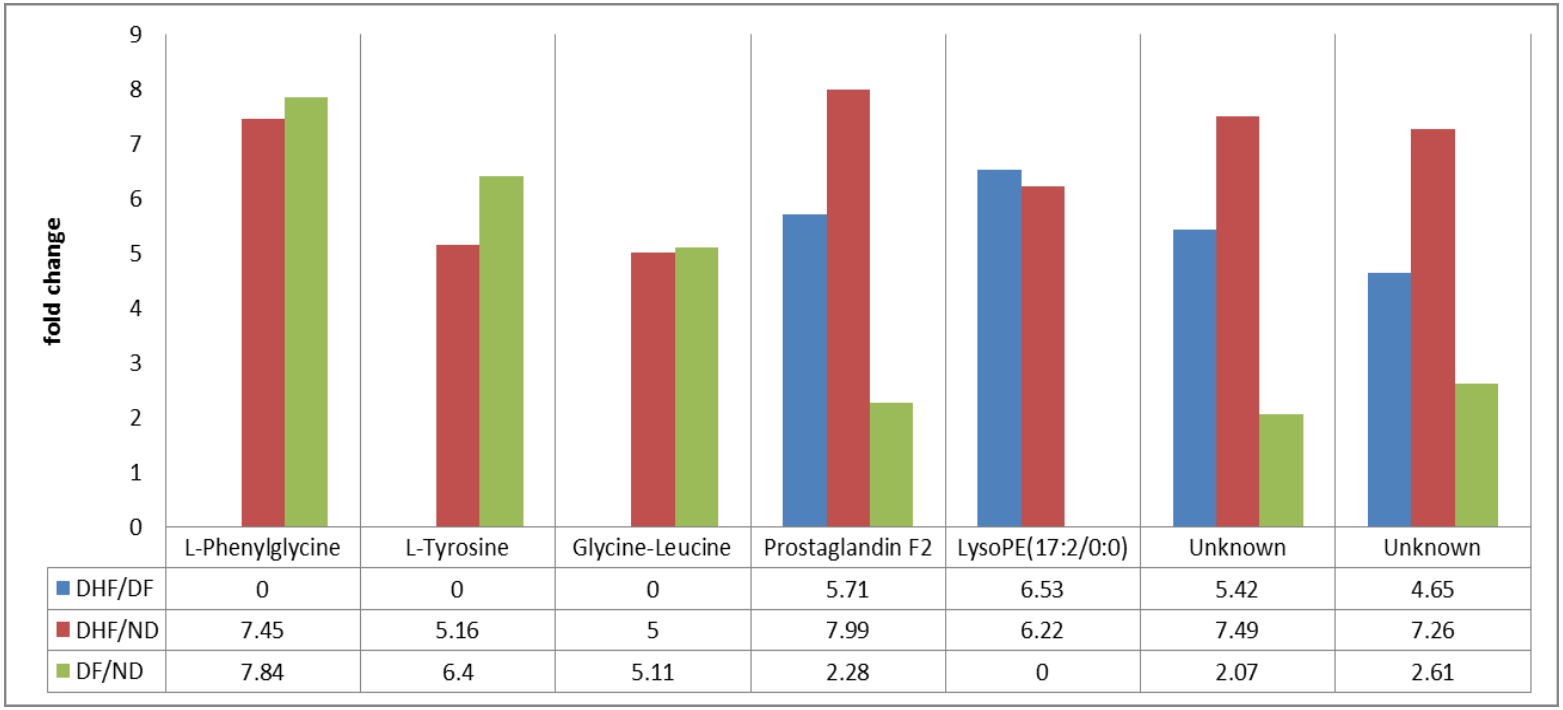
	Unknown	Alpha-linoleoyl choline	Prostaglandin D3	Hydroxy prostaglandin F2	Prostaglandin F2α	LysoPC(O-16:2/0:0)	LysoPC(18:0/0:0)	LysoPS(P-16:0/12:0)	PC(14:0/18:2)	PC(15:0/19:3)	PS(22:0/12:0)	PC(16:0/20:4)	PC(16:0/22:6)	PC(18:0/22:6)	PC(16:0/0:0)	PC(18:1/0:0)	Ceramide	Vitamin D3	25-hydroxy vitamin D3	25-dihydroxyvitamin D3
DHF/DF	-4.74	5.02	0	0	6.82	-3.66	0	0	3.44	4.79	-3.56	3.45	2.54	2.57	0	0	-1.83	-1.51	-1.84	0
DHF/ND	-12.4	8.87	-7.98	-7.85	10.59	-6.92	10.67	-8.66	8.66	10.51	-9.63	11.95	11.26	9.79	11.69	9.6	-7.1	-6.53	-7.64	-8.3
DF/ND	-7.65	3.85	-7.98	-7.85	3.77	-3.26	10.2	-8.66	5.22	5.72	-6.06	8.5	8.72	7.22	10.07	8.49	-5.28	-5.02	-5.79	-8.08

Figure 23. Selected candidate compounds detected in Mexican serum specimens by HILIC LC-MS analysis that differentiate the DHF/DSS, DF, and ND diagnostic groups. Figure presents selected candidate compounds and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents the respective pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

Table 22 .Selected candidate compounds detected in Mexican serum specimens by HILIC LC-MS analysis that differentiate the DHF/DSS, DF, and ND diagnostic groups*

Mexican serum			Calculated formula	# DB hits	Detected in samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
Mass	RT	Potential compound			% DHF	% DF	% ND	P value	FC	P value	FC	P value	FC
Unknowns													
226.1682	7.64	Unknown	C17H22	2	0	26	69	7.77E-03	-4.74	5.90E-03	-12.4	3.64E-08	-7.65
Fatty acids													
366.3293	1.29	Alpha-linoleoylcholine	C23H44NO2	1	57	30	6	4.30E-02	5.02	1.06E-03	8.87	9.04E-02	3.85
Eicosanoids													
350.2098	1.10	Prostaglandin D3	C20H30O5	44	0	0	50	_	_	9.28E-06	-7.98	4.04E-05	-7.98
370.2348	1.21	Hydroxy prostaglandin F2	C20H34O6	18	0	0	50	_	_	1.03E-05	-7.85	4.41E-05	-7.85
410.2585	1.36	Prostaglandin F2α	C23H38O6	3	65	26	6	7.06E-03	6.82	7.21E-05	10.59	9.93E-02	3.77
Phospholipids													
477.3211	14.55	LysoPC(O-16:2/0:0)	C24H48NO6P	3	17	41	63	7.66E-02	-3.66	3.52E-03	-6.92	1.95E-01	-3.26
523.365	14.72	LysoPC(18:0/0:0)	C26H54NO7P	15	52	48	0	_	_	5.71E-04	10.67	2.44E-04	10.20
663.4458	12.99	LysoPS(P-16:0/12:0)	C34H66NO9P	2	0	0	56	_	_	1.04E-06	-8.66	5.98E-06	-8.66
729.5309	12.96	PC(14:0/18:2)	C40H76NO8P	41	52	30	0	1.53E-01	3.44	1.56E-02	8.66	2.45E-04	5.22
755.5477	12.98	PC(15:0/19:3)	C42H78NO8P	45	52	30	0	8.73E-02	4.79	1.61E-02	10.51	2.52E-04	5.72
763.5513	11.72	PS(22:0/12:0)	C40H78NO10P	20	17	41	75	7.81E-02	-3.56	6.81E-05	-9.63	1.53E-02	-6.06
781.5647	12.57	PC(16:0/20:4)	C44H80NO8P	69	52	37	0	2.95E-01	3.45	4.70E-03	11.95	2.38E-04	8.50
805.5636	12.46	PC(16:0/22:6)	C46H80NO8P	62	52	41	0	4.15E-01	2.54	2.50E-03	11.26	2.40E-04	8.72
833.5936	12.37	PC(18:0/22:6)	C48H84NO8P	49	52	37	0	3.51E-01	2.57	4.74E-03	9.79	2.45E-04	7.22
495.3337	14.97	LysoPC(16:0/0:0)	C24H50NO7P	11	52	44	0	_	_	1.22E-03	11.69	2.42E-04	10.07
521.3493	14.8	LysoPC(18:1/0:0)	C26H52NO7P	19	52	44	0	_	_	1.24E-03	9.60	2.47E-04	8.49
Sphingolipids													
647.6207	1.30	Ceramide	C42H81NO3	3	26	44	69	_	_	2.35E-03	-7.10	0.01988	-5.28
Vitamin D derivatives													
384.3387	1.14	Vitamin D3	C27H44O	47	57	67	94	_	_	2.27E-02	-6.53	6.53E-03	-5.02
400.3339	1.16	25-hydroxyvitamin D3	C27H44O2	54	57	67	94	_	_	1.25E-02	-7.64	2.16E-03	-5.79
416.3275	1.31	25-dihydroxyvitamin D3	C27H44O3	70	43	44	94	-	-	3.06E-04	-8.30	2.52E-04	-8.08

* The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database during the *in silico* identification. The percentage of detection in each diagnosis group is a proportion of samples in which the compound was detected. The statistical differences determined by pairwise comparisons among diagnosis groups are provided. Missing values were not statistically significant. Abbreviations used are: RT-retention time, FC-fold change, DB-METLIN database, PS-phosphoserine, PI- phosphoinositol, PC-phosphatidylcholine, DHF-dengue hemorrhagic fever, DF-dengue fever, ND-non-dengue.



	L-Phenylglycine	L-Tyrosine	Glycine-Leucine	Prostaglandin F2	LysoPE(17:2/0:0)	Unknown	Unknown
DHF/DF	0	0	0	5.71	6.53	5.42	4.65
DHF/ND	7.45	5.16	5	7.99	6.22	7.49	7.26
DF/ND	7.84	6.4	5.11	2.28	0	2.07	2.61

Figure 24. Selected candidate compounds detected in Mexican serum specimens by RP-LC-MS analysis that differentiate the DHF/DSS, DF, and ND diagnostic groups. Figure presents selected candidate compounds and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents the respective pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

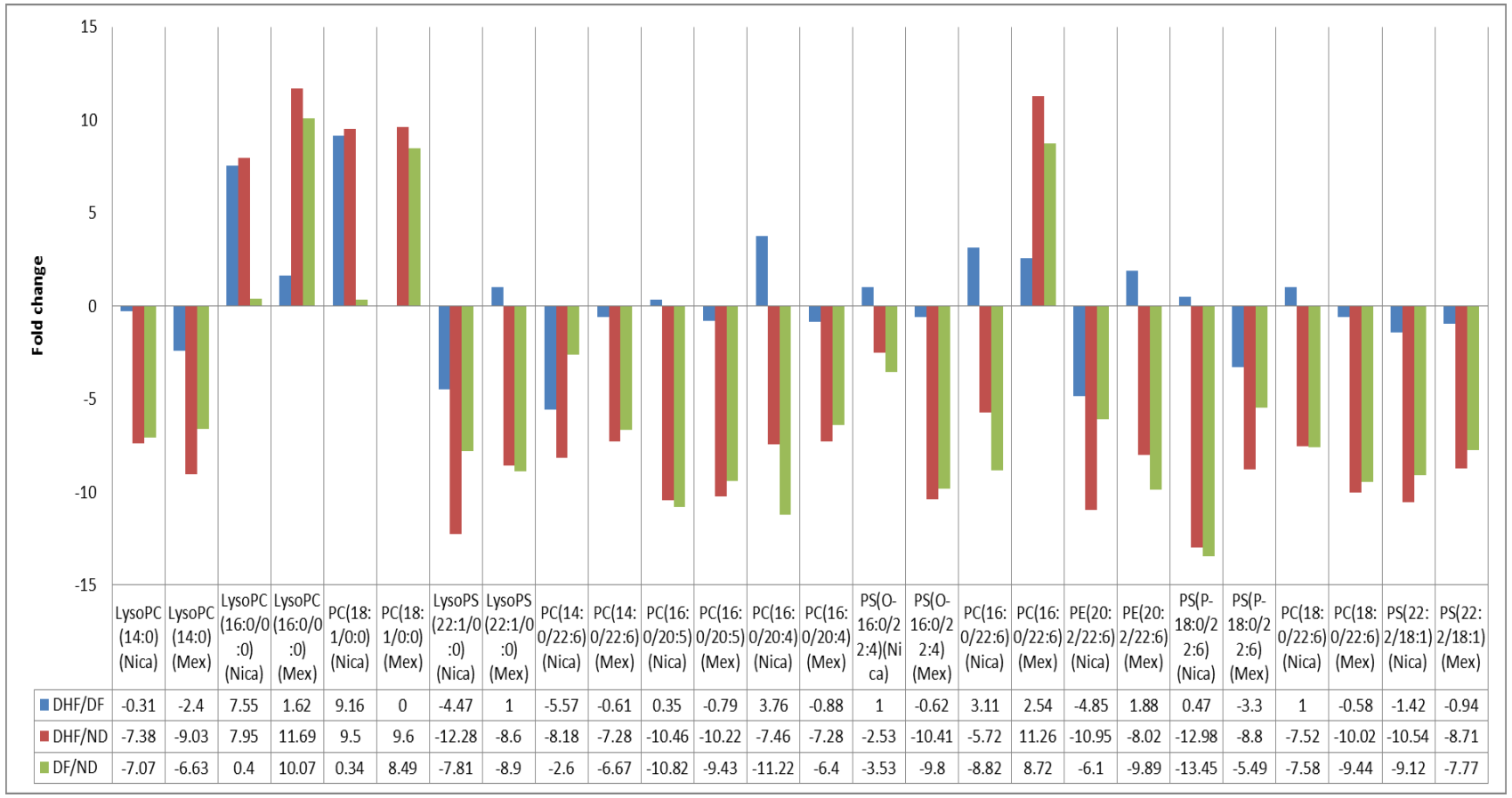
Table 23 . Selected candidate compounds detected in Mexican serum specimens by RP-LC-MS analysis that differentiate the DHF/DSS,DF and ND diagnostic groups*

Mexican serum RP			Calculated formula	#DB hits	Detected in Samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
MASS	RT	Potential compounds			% DHF	% DF	% ND	P value	FC	P value	FC	P value	FC
Amino acids and peptides													
151.0632	1.97	L-Phenylglycine	C8H9NO2	16	73	74	33	_	_	4.67E-03	7.45	2.81E-03	7.84
181.0739	3.34	L-Tyrosine	C9H11NO3	8	50	59	17	_	_	1.60E-02	5.16	2.59E-03	6.40
188.1164	1.01	Glycine-Leucine	C8H16N2O3	23	50	52	11	_	_	6.92E-03	5.00	4.94E-03	5.11
Eicosanoids													
410.2582	16.78	Prostaglandin F2	C23H38O6	3	50	19	6	1.48E-02	5.71	1.51E-03	7.99	0.22	2.28
Phospholipids													
463.2755	10.68	LysoPE(17:2/0:0)	C22H42NO7P	2	62	19	22	6.77E-04	6.53	4.60E-03	6.22	_	_
Unknown													
567.4409	16.79	Unknown	C37H59O	0	50	19	6	1.20E-02	5.42	1.33E-03	7.49	0.23	2.07
563.2678	16.81	Unknown	C26H45NO8S2	2	50	19	0	1.26E-02	4.65	1.66E-04	7.26	5.45E-02	2.61

* The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database during the *in silico* identification. The percentage of detection in each diagnosis group is a proportion of samples in which the compound was detected. The statistical differences determined by pairwise comparisons among diagnosis groups are provided. Missing values were not statistically significant. Abbreviations used are: RT-retention time, FC-fold change, DB-METLIN database, PE-phosphoethanolamine, DHF-dengue hemorrhagic fever, DF-dengue fever, ND-non-dengue.

The most numerous and biologically and statistically relevant groups of compounds were those associated with fatty acids and phospholipids (Table 24). In serum specimens from patients in both countries, the majority of phospholipids had a negative FC in DHF/DSS patients (Figure 25). Due to the biological significance of lipids in DEN disease, [8] the statistically significant potential phospholipids found in serum are presented by country of origin in Table 24.

Biologically relevant compounds identified as phospholipids that differentiated the DHF/DSS from DF patients in both the Nicaraguan and Mexican serum specimens included 1-palmitoyl-lysophosphatidylcholine and 1-oleoyl- lysophosphatidylcholine, (listed in Table 24 as LysoPC (16:0/0:0) and LysoPC (18:1/0:0) respectively) which had a positive FC in DHF/DSS vs. DF and DHF/DSS vs. ND in Nicaraguan patients but were increased in DHF/DSS vs. ND and DF vs. ND but not in DHF/DSS vs. DF in Mexican patients. These could be interesting candidates to pursue as biomarkers for diagnosis or prognosis of DEN disease outcomes.



	LysoPC (14:0) (Nica)	LysoPC (14:0) (Mex)	LysoPC (16:0/0 :0) (Nica)	LysoPC (16:0/0 :0) (Mex)	PC(18: 1/0:0) (Nica)	PC(18: 1/0:0) (Mex)	LysoPS (22:1/0 :0) (Nica)	LysoPS (22:1/0 :0) (Mex)	PC(14: 0/22:6) (Nica)	PC(14: 0/22:6) (Mex)	PC(16: 0/20:5) (Nica)	PC(16: 0/20:5) (Mex)	PC(16: 0/20:4) (Nica)	PC(16: 0/20:4) (Mex)	PS(O- 16:0/2 2:4)(Ni ca)	PS(O- 16:0/2 2:4) (Mex)	PC(16: 0/22:6) (Nica)	PC(16: 0/22:6) (Mex)	PE(20: 2/22:6) (Nica)	PE(20: 2/22:6) (Mex)	PS(P- 18:0/2 2:6) (Nica)	PS(P- 18:0/2 2:6) (Mex)	PC(18: 0/22:6) (Nica)	PC(18: 0/22:6) (Mex)	PS(22: 2/18:1) (Nica)	PS(22: 2/18:1) (Mex)
DHF/DF	-0.31	-2.4	7.55	1.62	9.16	0	-4.47	1	-5.57	-0.61	0.35	-0.79	3.76	-0.88	1	-0.62	3.11	2.54	-4.85	1.88	0.47	-3.3	1	-0.58	-1.42	-0.94
DHF/ND	-7.38	-9.03	7.95	11.69	9.5	9.6	-12.28	-8.6	-8.18	-7.28	-10.46	-10.22	-7.46	-7.28	-2.53	-10.41	-5.72	11.26	-10.95	-8.02	-12.98	-8.8	-7.52	-10.02	-10.54	-8.71
DF/ND	-7.07	-6.63	0.4	10.07	0.34	8.49	-7.81	-8.9	-2.6	-6.67	-10.82	-9.43	-11.22	-6.4	-3.53	-9.8	-8.82	8.72	-6.1	-9.89	-13.45	-5.49	-7.58	-9.44	-9.12	-7.77

Figure 25. Selected phospholipids that were detected in both Nicaraguan and Mexican serum samples that differentiate disease diagnosis groups. Figure presents selected phospholipids and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents the respective pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

Table 24. Selected phospholipids that were detected in Nicaraguan (white) and Mexican (green) serum samples that differentiate diagnosis groups*

Selected phospholipids			Calculated formula	# DB hits	Detected in samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
Mass	RT	Potential compounds			% DHF	% DF	% ND	P value	FC	P value	FC	P value	FC
Phospholipids													
467.3008	13.97	LysoPC(14:0)	C22H46NO7P	14	12	13	58	8.36E-01	-0.31	2.63E-04	-7.38	8.78E-04	-7.07
467.3011	14.89				39	52	88	3.36E-01	-2.40	8.62E-04	-9.03	1.23E-02	-6.63
495.3329	13.66	LysoPC(16:0/0:0) or 1-palmitoyl-lysophosphatidyl-choline	C24H50NO7P	11	65	29	27	9.29E-03	7.55	4.97E-03	7.95	8.83E-01	0.40
495.3337	14.96				52	44	0	_	1.62	1.22E-03	11.69	2.42E-04	10.07
521.348	13.73	LysoPC(18:1(9Z)/0:0) or 1-oleoyl-lysophosphatidylcholine	C26H52NO7P	19	62	17	15	1.02E-03	9.16	4.03E-04	9.50	8.78E-01	0.34
521.3493	14.8				52	44	0	_	_	1.24E-03	9.60	2.47E-04	8.49
579.3536	13.2	LysoPS(22:1(11Z)/0:0)	C28H54NO9P	1	15	46	92	1.88E-02	-4.47	4.19E-12	-12.28	4.54E-05	-7.81
579.3556	13.92				9	7	63	7.84E-01	1	8.86E-05	-8.60	8.54E-06	-8.90
777.5311	11.86	PC(14:0/22:6)	C44H76NO8P	48	50	79	96	9.06E-03	-5.57	1.13E-05	-8.18	1.01E-01	-2.60
777.5327	11.78				30	33	75	7.75E-01	-0.61	3.99E-03	-7.28	7.53E-03	-6.67
779.5478	11.87	PC(16:0/20:5)	C44H78NO8P	43	31	25	85	8.70E-01	0.35	2.86E-06	-10.46	1.83E-06	-10.82
779.5471	11.83				9	11	56	6.16E-01	-0.79	2.07E-04	-10.22	6.97E-04	-9.43
781.5627	12.03	PC(16:0/20:4)	C44H80NO8P	69	38	17	81	6.82E-02	3.76	8.63E-04	-7.46	1.16E-07	-11.22
781.5653	12.04				17	41	38	7.30E-01	-0.88	1.01E-02	-7.28	2.48E-02	-6.40
797.558	11.92	PS(O-16:0/22:4)	C44H80NO9P	10	96	96	100	4.09E-01	1.00	5.45E-03	-2.53	1.25E-04	-3.53
797.5585	11.94				30	33	75	8.23E-01	-0.62	2.17E-03	-10.41	2.95E-03	-9.80
805.5633	11.78	PC(16:0/22:6)	C46H80NO8P	62	31	13	65	1.09E-01	3.11	1.24E-02	-5.72	3.95E-05	-8.82
805.5631	11.73				43	52	88	5.54E-01	2.54	3.33E-03	11.26	1.36E-02	8.72
815.567	11.49	PE(20:2(11Z,14Z)/22:6)	C47H78NO8P	40	38	71	92	1.91E-02	-4.85	2.76E-08	-10.95	9.76E-04	-6.10
815.569	11.67				13	4	56	1.92E-01	1.88	3.67E-03	-8.02	1.27E-05	-9.89
819.5418	11.83	PS(P-18:0/22:6)	C48H84NO8P	1	8	4	77	6.48E-01	0.47	1.15E-09	-12.98	3.96E-10	-13.45
819.5461	12.31				4	26	56	3.83E-02	-3.30	6.78E-05	-8.80	2.63E-02	-5.49
833.5939	12.40	PC(18:0/22:6)	C48H84NO8P	49	31	29	65	9.85E-01	1	1.07E-02	-7.52	1.28E-02	-7.58
833.5926	11.75				35	48	63	8.46E-01	-0.58	3.12E-03	-10.02	4.55E-03	-9.44
841.5815	11.34	PS(22:2/18:1)	C46H84NO10P	18	15	25	77	4.01E-01	-1.42	2.18E-07	-10.54	2.56E-05	-9.12
841.5825	11.47				26	33	69	6.71E-01	-0.94	2.87E-03	-8.71	5.17E-03	-7.77

*The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database during the *in silico* identification. The percentage of detection in each diagnosis group is a proportion of samples in which the compound was detected. The statistical differences determined by pairwise comparisons among diagnosis groups are provided. Missing values were not statistically significant. Abbreviations used are: RT-retention time, FC-fold change, DB-METLIN database, PS-phosphoserine, PC-phosphatidylcholine, PE-phosphoethanolamine, DHF-dengue hemorrhagic fever, DF-dengue fever, ND-non-dengue.

4.D.3.c. Results with Nicaraguan urine specimens. In urine specimens from Nicaraguan patients, a list of twenty compounds (Table 25) that differed statistically in the pairwise comparison of DEN diagnosis group was selected from the molecular features listed in Table 19 based on their biological significance and their potential role in DEN pathogenesis, as well as their satisfactory statistical values in pairwise comparisons that differentiated the disease diagnosis groups. These compounds are presented in Figure 26, which represents the positive or negative FC of each compound in pairwise comparisons of DEN diagnosis.

Twelve candidate compounds differed significantly between the DHF/DSS and DF diagnosis groups, eleven differed significantly between the DHF/DSS and ND diagnosis groups, and three differed significantly for DF and ND diagnosis groups (Table 25, Figure 26).

A visual representation of the positive and negative FC obtained in this group of clinical samples is presented in Figure 26.

The biologically relevant compounds were divided into their correspondent biochemical classes. In Nicaraguan urine samples, the candidate compounds were classified into groups that included nucleosides, amino acids, peptides and derivatives and unknowns (Table 25).

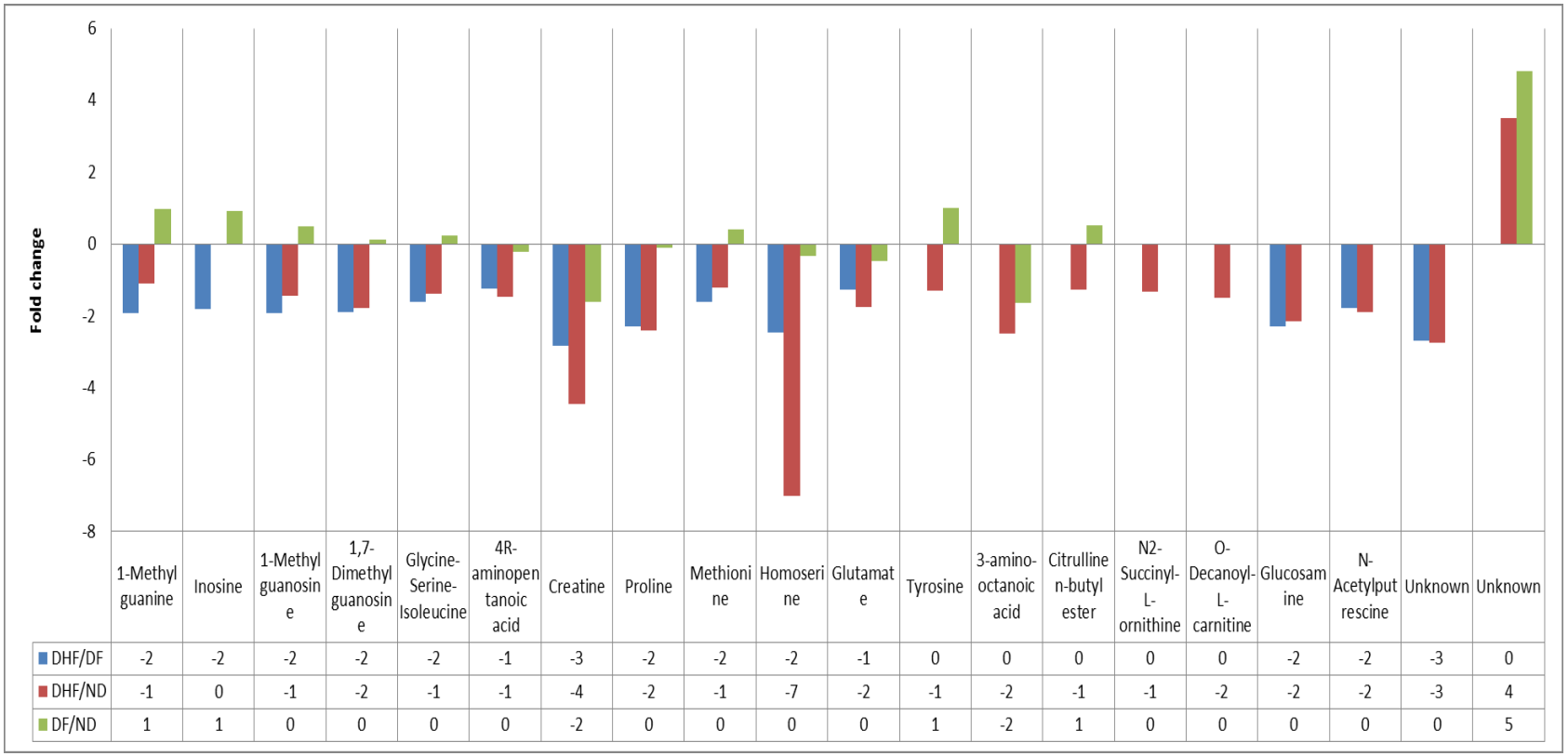
Pairwise comparisons of compounds that differentiated the DHF/DSS and DF diagnosis groups yielded P-values that ranged from 9.48E-05 and a FC of -2.82 of potentially identified creatine, to potential methionine with a P-value of 3.36E-04 and a FC of -1.61 that also appeared reduced according to the negative FC (Table 25).

The pairwise comparison of compounds that differentiated of the DHF/DSS from ND diagnosis groups in Nicaraguan urine samples yielded statistical values ranging from 1.77E-09 and a FC of -4.44 for the potentially identified creatine, to 7.84E-06 and FC of 3.5O for an unknown compound.

The pairwise comparisons of compounds that differentiated DF from ND diagnosis groups yielded P-values that ranged from 3.31E-11 and a FC of -1.63 for potentially 3-amino-octanoic acid to an unknown compound that had a FC of 4.81 (Figure 26, Table 25).

In general, the FC observed in Nicaraguan urine specimens were very low compared to those detected in Mexican specimens, and several of the selected features did not have a FC of >2. These compounds were selected based on significant P-values and a FC of >1.

The Nicaraguan urine samples showed consistently negative FC in the majority of the compounds (Figure 26, Table 25) when compared to Mexican urine specimens, which suggests that the sample could have been compromised during collection, handling or shipment and that the quality of the data obtained may not be optimal.



	1-Methyl guanine	Inosine	1-Methyl guanosine	1,7-Dimethyl guanosine	Glycine-Serine-Isoleucine	4R-aminopentanoic acid	Creatine	Proline	Methionine	Homoserine	Glutamate	Tyrosine	3-amino-octanoic acid	Citrulline-butyl ester	N2-Succinyl-L-ornithine	O-Decanoyl-L-carnitine	Glucosamine	N-Acetylputrescine	Unknown	Unknown
DHF/DF	-2	-2	-2	-2	-2	-1	-3	-2	-2	-2	-1	0	0	0	0	0	-2	-2	-3	0
DHF/ND	-1	0	-1	-2	-1	-1	-4	-2	-1	-7	-2	-1	-2	-1	-1	-2	-2	-2	-3	4
DF/ND	1	1	0	0	0	0	-2	0	0	0	0	1	-2	1	0	0	0	0	0	5

Figure 26. Selected candidate compounds detected in Nicaraguan urine specimens by HILIC-LC-MS analysis that differentiate the DHF/DSS, DF, and ND diagnostic groups. Figure presents selected candidate compounds and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents the respective pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

Table 25 . Selected candidate compounds detected in Nicaraguan surine specimens that differentiate the DHF/DSS, DF, and ND diagnostic groups by HLIC LC-MS analysis*

Nicaraguan urine samples			Calculated formula	#DB hits	Detected in samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
Mass	RT	Potential compound			% DHF	% DF	% ND	P val	FC	P val	FC	P val	FC
Nucleosides													
165.0652	9.58	1-Methylguanine	C6H7N5O	5	50	47	39	7.86E-08	-1.93	2.34E-03	-1.11	-	0.98
268.0804	5.32	Inosine	C10H12N4O5	35	36	39	42	2.03E-07	-1.81	-	-	-	0.93
297.1078	6.29	1-Methylguanosine	C11H15N5O5	11	64	53	71	5.98E-06	-1.91	1.28E-03	-1.43	1.51E-02	0.48
311.1227	6.93	1,7-Dimethylguanosine	C12H17N5O5	16	57	64	65	3.35E-04	-1.90	1.76E-03	-1.77	-	0.13
Amino acids, peptides and derivatives													
275.1467	22.71	Glycine-Serine-Isoleucine	C11H21N3O5	41	50	22	23	6.30E-06	-1.62	3.30E-04	-1.38	-	0.24
117.0789	15.39	4R-aminopentanoic acid	C5H11NO2	17	29	47	52	5.04E-05	-1.25	1.23E-07	-1.48	-	-0.23
131.0692	14.45	Creatine	C4H9N3O2	3	50	31	26	9.48E-05	-2.82	1.77E-09	-4.44	3.35E-02	-1.62
115.0614	3.02	Proline	C5H9NO2	7	43	39	65	2.19E-04	-2.29	1.49E-04	-2.40	-	-0.11
149.0508	8.30	Methionine	C5H11NO2S	11	64	89	81	3.36E-04	-1.61	3.85E-03	-1.20	-	0.41
119.0586	7.26	Homoserine	C4H9NO3	7	43	47	42	1.68E-03	-2.45	5.76E-04	-7	-	-0.32
147.0566	2.10	Glutamate	C5H9NO4	13	57	8	13	9.77E-03	-1.27	6.32E-04	-1.74	-	-
181.0742	5.26	Tyrosine	C9H11NO3	8	50	42	45	-	-	1.91E-03	-1.30	1.83E-03	1
159.1259	17.83	3-amino-octanoic acid	C8H17NO2	7	43	14	6	-	-	1.59E-06	-2.48	3.31E-11	-1.63
231.1577	5.54	Citrulline n-butyl ester	C10H21N3O3	10	50	44	58	-	-	4.40E-03	-1.28	-	0.53
232.1025	4.19	N2-Succinyl-L-ornithine	C9H16N2O5	15	29	42	26	-	-	3.59E-03	-1.32	-	-
315.2367	5.00	O-Decanoyl-L-carnitine	C17H33NO4		57	44	48	-	-	3.90E-03	-1.50	-	-
Other													
179.0806	6.93	Glucosamine	C6H13NO5	8	71	53	58	1.96E-06	-2.30	4.20E-05	-2.14	-	-
130.1107	19.82	N-Acetylputrescine	C6H14N2O	2	36	53	35	8.06E-04	-1.79	9.19E-04	-1.88	-	-
Unknown													
285.1929	10.91	Unknown	C13H25N4O3	0	29	67	39	1.70E-03	-2.69	1.11E-03	-2.74	-	-
601.5662	6.05	Unknown	C37H71N5O	0	43	6	10	-	-	7.84E-06	3.50	0	4.81

* The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database during the *in silico* identification. The percentage of detection in each diagnosis group is a proportion of samples in which the compound was detected. The statistical differences determined by pairwise comparisons among diagnosis groups are provided. Missing values were not statistically significant. Abbreviations used are: RT-retention time, FC-fold change, DB-METLIN database, DHF-dengue hemorrhagic fever, DF-dengue fever, ND-non-dengue.

4.D.3.d. Results with Mexican urine specimens. LC-MS analysis of the Mexican urine specimens revealed several molecular features that statistically differentiated the DEN diagnosis listed in Table 19 and Appendices 4 and 5.

Compounds were further selected based on their biological significance and their potential role in DEN pathogenesis, as well as their statistical values in pairwise comparisons that differentiated the disease diagnosis groups.

In Mexican urine specimens several of the selected features did not had a FC of >2, instead were selected based on significant P-values and FC of >1 (Appendices 4 and 5). These compounds are presented in Figure 27, which represents the positive or negative FC of each compound in pairwise comparisons of DEN diagnosis.

Thirteen compounds were selected that differed statistically among the diagnosis groups (Table 26). Ten compounds differentiated DHF/DSS and DF patients, eleven differentiated DHF/DSS and ND patients, and one differentiated DF and ND patients (Figure 27, Table 26).

In the Mexican urine samples, the classes of compounds included nucleosides, amino acids, peptides and derivatives, and vitamin D derivatives.

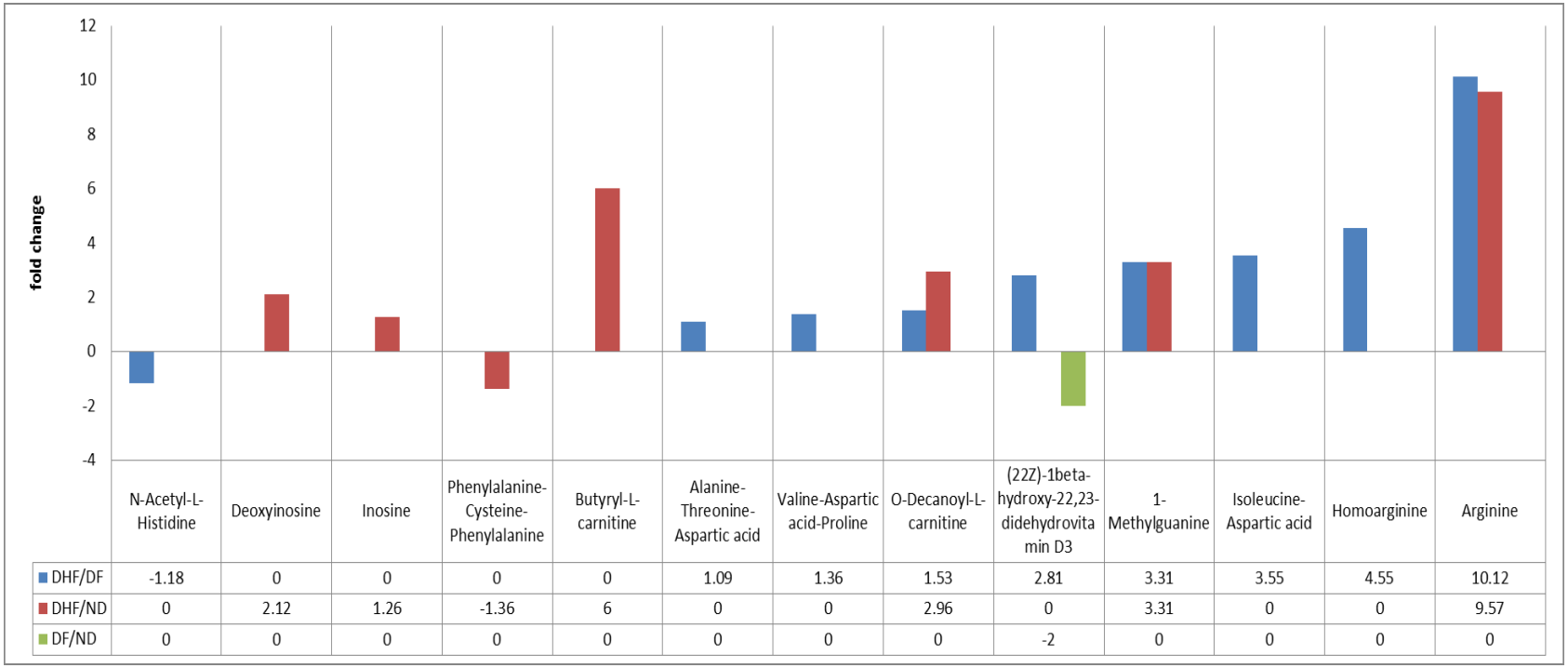
Pairwise comparisons of compounds that differentiated the DHF/DSS and DF diagnosis groups yielded P-values that ranged from value of 4.94E-03 and FC of -1.18 for a potential histidine isotype that is decreased in DHF/DSS, to a P-value of 3.68E-05 and a FC of 10.12 for a potential amino acid arginine, which was importantly increased (Table 26).

The pairwise comparison of compounds that differentiated of the DHF/DSS from ND diagnosis groups in Mexican urine samples yielded statistical values that ranged from P-value of 1.00E-07 with a FC of 2.12 for potentially identified deoxyinosine to potentially identified arginine with a P-value of 3.49E-05 and a FC of 9.57.

For the pairwise comparison of DF from ND samples, only one compound distinguished diagnosis groups and had a P-value of 2.06E-09 and a FC of -2 and is potentially a vitamin D3 isotype (Figure 27, Table 26).

Some relevant candidate compounds among Nicaraguan and Mexican urine specimens that differentiate diagnosis groups but whose FC and P-values are not as great as those mentioned above for DHF/DSS from DF in both the Nicaraguan and Mexican urine include: 1-methylguanine, and decanoyl-carnitine.

The regulation, either increased or decreased for each compound detected among countries is consistently different although the P-values are significant. This could be due to inherent differences among the samples, or be caused by sample collection and handling (Tables 25 and 26).



	N-Acetyl-L-Histidine	Deoxyinosine	Inosine	Phenylalanine-Cysteine-Phenylalanine	Butyryl-L-carnitine	Alanine-Threonine-Aspartic acid	Valine-Aspartic acid-Proline	O-Decanoyl-L-carnitine	(22Z)-1beta-hydroxy-22,23-didehydrovitamin D3	1-Methylguanine	Isoleucine-Aspartic acid	Homoarginine	Arginine
DHF/DF	-1.18	0	0	0	0	1.09	1.36	1.53	2.81	3.31	3.55	4.55	10.12
DHF/ND	0	2.12	1.26	-1.36	6	0	0	2.96	0	3.31	0	0	9.57
DF/ND	0	0	0	0	0	0	0	0	-2	0	0	0	0

Figure 27. Selected candidate compounds detected in Mexican urine specimens by HILIC-LC-MS analysis that differentiate the DHF/DSS, DF, and ND diagnostic groups. Figure presents selected candidate compounds and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents the respective pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

Table 26 . Selected canddiate compounds detected in Mexican urine specimens that differentiate the DHF/DSS, DF, and ND diagnostic groups by HLIC LC-MS analysis*

Mexican urine samples			Calculated formula	#DB hits	Detected in samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
Mass	RT	Potential compound			% DHF	% DF	% ND	P val	FC	P val	FC	P val	FC
Nucleosides													
165.065	9.29	1-Methylguanine	C6H7N5O	5	55	69	71	3.38E-03	3.31	2.70E-03	3.31	-	-
252.086	3.41	Deoxyinosine	C10H12N4O4	18	45	78	38	-	-	1.00E-07	2.12	-	-
268.081	5.37	Inosine	C10H12N4O5	49	36	38	38	-	-	1.20E-04	1.26	-	-
Amino acids, peptides and derivatives													
174.112	23.49	Arginine	C6H14N4O2	8	9	22	35	3.68E-05	10.12	3.49E-05	9.57	-	-
188.127	5.84	Homoarginine	C7H16N4O2	6	45	59	59	1.65E-03	4.55	-	-	-	-
197.082	16.59	N-Acetyl-L-Histidine	C8H11N3O3	6	14	41	62	4.94E-03	-1.18	-	-	-	-
246.123	12.31	Isoleucine-Aspartic acid	C10H18N2O5	45	36	25	35	6.01E-03	3.55	-	-	-	-
305.123	1.26	Alanine-Threonine-Aspartic acid	C11H19N3O7	23	45	53	47	2.55E-04	1.09	-	-	-	-
329.159	4.54	Valine-Aspartic acid-Proline	C14H23N3O6	16	36	16	44	4.27E-04	1.36			-	-
415.158	5.13	Phenylalanine-Cysteine-Phenylalanine	C21H25N3O4S1	11	14	31	50	-	-	3.89E-14	-1.36		
231.147	11.24	Butyryl-L-carnitine	C11H21NO4	4	73	81	74	-	-	1.66E-03	6	-	-
315.239	4.6	O-Decanoyl-L-carnitine	C17H33NO4	4	18	28	38	2.79E-04	1.53	2.27E-03	2.96	-	-
Vitamin D derivatives													
398.32	1.35	1beta-hydroxy-22,23-didehydrovitamin D3	C27H42O2	16	50	28	41	5.67E-03	2.81	-	-	2.06E-09	-2

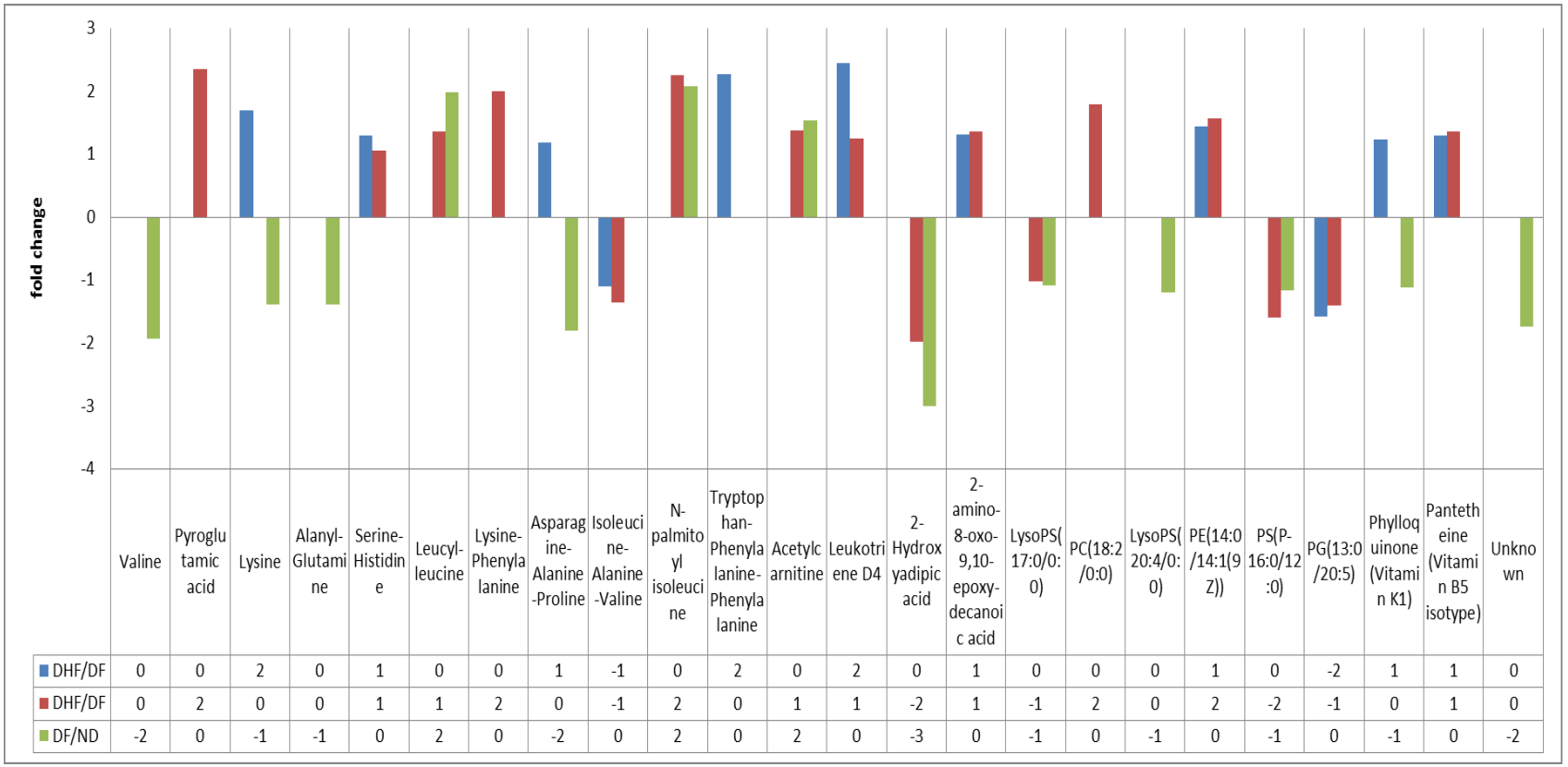
* The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database during the *in silico* identification. The percentage of detection in each diagnosis group is a proportion of samples in which the compound was detected. The statistical differences determined by pairwise comparisons among diagnosis groups are provided. Missing values were not statistically significant. Abbreviations used are: RT-retention time, FC-fold change, DB-METLIN database, DHF-dengue hemorrhagic fever, DF-dengue fever, ND-non-dengue.

4.D.3.e. Results with Nicaraguan saliva specimens. In Nicaraguan saliva, a list of twenty-four compounds that had statistical differences in the pairwise comparison of DEN diagnosis group was selected from the molecular features listed in Table 20 based on their biological significance and their potential role in DEN pathogenesis, as well as their satisfactory statistical values in pairwise comparisons that differentiated the disease diagnosis groups. These compounds are presented in Figure 28, which represents the positive or negative FC of each compound in pairwise comparisons of DEN diagnosis.

The selected compounds that differentiated DEN disease outcomes were from six major biochemical groups. These included amino acids, peptides and derivatives, eicosanoids, fatty acids derivatives, phospholipids, vitamin D derivatives and unidentified. Four compounds differentiated DHF/DSS from DF, seven differentiated for DHF/DSS from ND, and seven differentiated DF from ND (Figure 28, Table 27). Pairwise comparisons of compounds that differentiated the DHF/DSS and DF diagnosis groups yielded P-values that ranged from 2.64E-08 and a FC of -1.58 of a potentially identified phosphatidylglycerol isotype to a P-value of 2.96E-04and a FC of 2.27 for potentially identified peptide consisting on tryptophan-phenylalanine-phenylalanine (Figure 28, Table 27).

The pairwise comparison of compounds that differentiated of the DHF/DSS from the ND diagnosis groups yielded statistical values ranging from the potentially identified as hydroxyadipic acid with a P-value of 3.09E-03 with a FC of -1.98 to a P-value of 2.41E-03 with a FC of 2.34 of pyroglutamic acid. The pairwise comparisons of compounds that differentiated DF from ND diagnosis groups yielded P-values that ranged from 2.51E-07 and a FC of -3 for hydroxyadipic acid isotype to a p value of 4.64E-11 with a FC of 1.98 for leucyl-leucine (Table 27).

The fold changes detected Nicaraguan saliva specimens were consistently smaller than in the Mexican saliva specimens and the selected features were selected based on significant P-values and FC of >1. This suggests that the sample could have been compromised during collection, handling or shipment and the quality of the data obtained for saliva may not be optimal.



	Valine	Pyroglutamic acid	Lysine	Alanyl-Glutamine	Serine-Histidine	Leucyl-leucine	Lysine-Phenylalanine	Asparagine-Alanine-Proline	Isoleucine-Alanine-Valine	N-palmitoyl isoleucine	Tryptophan-Phenylalanine-Phenylalanine	Acetylcarnitine	Leukotriene D4	2-Hydroxyadipic acid	2-amino-8-oxo-9,10-epoxy-decanoic acid	LysoPS(17:0/0:0)	PC(18:2/0:0)	LysoPS(20:4/0:0)	PE(14:0/14:1(9Z))	PS(P-16:0/12:0)	PG(13:0/20:5)	Phylloquinone (Vitamin K1)	Pantetheine (Vitamin B5 isotype)	Unknown
DHF/DF	0	0	2	0	1	0	0	1	-1	0	2	0	2	0	1	0	0	0	1	0	-2	1	1	0
DHF/DF	0	2	0	0	1	1	2	0	-1	2	0	1	1	-2	1	-1	2	0	2	-2	-1	0	1	0
DF/ND	-2	0	-1	-1	0	2	0	-2	0	2	0	2	0	-3	0	-1	0	-1	0	-1	0	-1	0	-2

Figure 28. Selected candidate compounds detected in Nicaraguan saliva specimens by HILIC-LC-MS that differentiate the DHF/DSS, DF, and ND diagnostic groups. Figure presents selected candidate compounds and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents the respective pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

Table 27 . Selected candidate compounds detected in Nicaraguan saliva specimens that differentiate the DHF/DSS, DF, and ND diagnostic groups by HLIC LC-MS analysis*

Nicaraguan saliva samples			Calculated formula	#DB hits	Presence in samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
Mass	**RT**	**Potential compound**			**% DHF**	**% DF**	**% ND**	**P val**	**FC**	**Pval**	**FC**	**Pval**	**FC**
Amino acids, peptides and derivatives													
117.0788	12.95	Valine	C5H11NO2	17	53	41	28	-	-	-	-	5.31E-04	-1.93
129.0426	1.73	Pyroglutamic acid	C5H7NO3	8	89	86	59	-	-	2.41E-03	2.34	-	-
146.1054	28.46	Lysine	C6H14N2O2	7	42	8	13	1.05E-05	1.69	-	-	2.13E-06	-1.39
217.1069	29.63	Alanyl-Glutamine	C8H15N3O4	14	42	27	22	-	-	-	-	1.61E-04	-1.39
242.1018	26.53	Serine-Histidine	C9H14N4O4	10	42	32	38	5.06E-04	1.30	3.17E-04	1.05	-	-
244.1787	12.86	Leucyl-leucine	C12H24N2O3	13	42	32	28	-	-	3.41E-04	1.36	4.64E-11	1.98
293.1742	27.74	Lysine-Phenylalanine	C15H23N3O3	11	74	62	47	-	-	2.71E-04	2	-	-
300.1500	30.23	Asparagine-Alanine-Proline	C12H20N4O5	14	47	24	9	9.26E-05	1.18	-	-	1.35E-05	-1.80
301.2001	13.86	Isoleucine-Alanine-Valine	C14H27N3O4	26	42	27	34	2.06E-03	-1.10	5.21E-04	-1.36	-	-
369.3244	1.22	N-palmitoyl isoleucine	C22H43NO3	5	37	49	47	-	-	7.51E-04	2.25	1.33E-03	2.07
498.2267	26.41	Tryptophan-Phenylalanine-Phenylalanine	C29H30N4O4	10	58	54	44	2.96E-04	2.27	-	-	-	-
203.1159	16.28	Acetylcarnitine	C9H17NO4	5	47	30	28	-	-	4.22E-04	1.37	1.00E-04	1.54
Eicosanoids													
496.2543	39.89	Leukotriene D4	C25H40N2O6S	3	53	43	38	3.95E-04	2.45	7.90E-03	1.25	-	-
Fatty acids derivatives													
162.0529	7.89	2-Hydroxyadipic acid	C6H10O5	26	42	46	25	-	-	3.09E-03	-1.98	2.51E-07	-3.00
215.1167	34.33	2-amino-8-oxo-9,10-epoxy-decanoic acid	C10H17NO4	2	42	19	16	2.75E-02	1.31	5.91E-05	1.36	-	-
Phospholipids													
511.3018	1.21	LysoPS(17:0/0:0)	C23H46NO9P	2	58	38	38	-	-	1.62E-03	-1.02	1.38E-05	-1.08
519.3256	9.25	PC(18:2/0:0)	C26H50NO7P	5	47	41	28	-	-	4.26E-03	1.79	-	-
545.2716	32.98	LysoPS(20:4/0:0)	C26H44NO9P	8	53	30	22	-	-	-	-	4.93E-03	-1.19
633.4456	1.53	PE(14:0/14:1(9Z))	C33H64NO8P	8	63	51	19	2.23E-05	1.44	1.05E-03	1.57	-	-
726.4532	2.43	PG(13:0/20:5)	C39H67O10P	8	42	32	22	2.64E-08	-1.58	2.15E-04	-1.41	-	-
Vitamin derivatives													
450.3545	1.08	Phylloquinone (Vitamin K1)	C31H46O2	1	47	30	41	9.59E-04	1.23	-	-	4.87E-03	-1.12
278.1310	33.57	Pantetheine (Vitamin B5)	C11H22N2O4S	11	58	41	47	2.95E-03	1.29	6.05E-05	1.36	-	-
Unknown													
506.2486	32.52	Unknown	C27H38O9	2	89	65	50	-	-	-	-	2.96E-03	-1.74

* The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database during the *in silico* identification. The percentage of detection in each diagnosis group is a proportion of samples in which the compound was detected. The statistical differences determined by pairwise comparisons among diagnosis groups are provided. Missing values were not statistically significant. Abbreviations used are: RT-retention time, FC-fold change, DB-METLIN database, PS-phosphoserine, PE- phosphoethanolamine, PG-phosphoglycerol, DHF-dengue hemorrhagic fever, DF-dengue fever, ND-non-dengue.

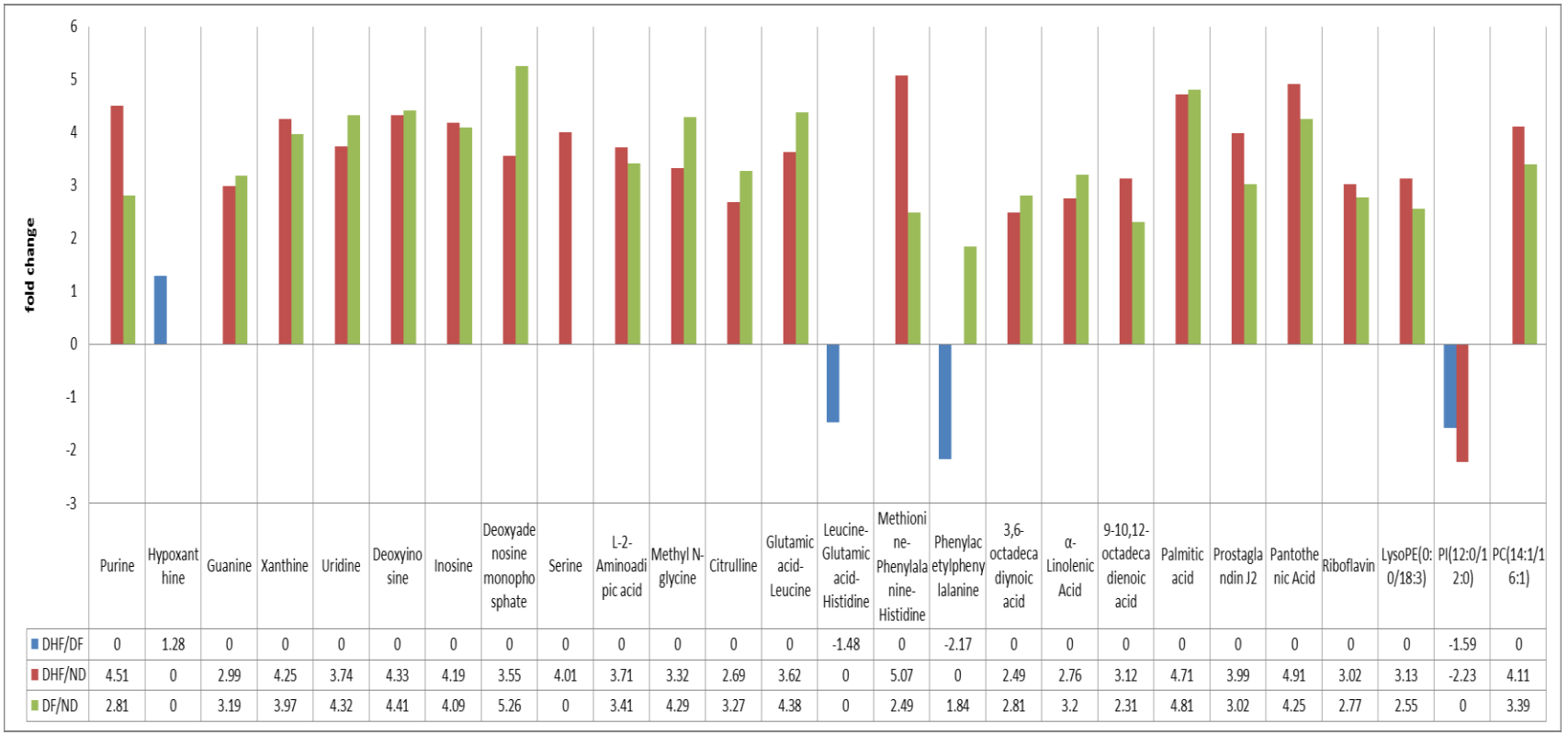
4.D.3.f. Results with Mexican saliva specimens. In Mexican saliva specimens, a list of twenty-six compounds that differed statistically in the pairwise comparison of DEN diagnosis group was selected based on their biological significance and their potential role in DEN pathogenesis, as well as their satisfactory statistical values in pairwise comparisons that differentiated the diagnosis groups. These compounds are presented in Figure 29, which represents the positive or negative FC of each compound in pairwise comparisons of DEN diagnosis.

The potential compounds that differentiated DEN disease groups found in the Mexican saliva were classified into major biochemical groups, which included nucleosides, amino acids, peptides and derivatives, fatty acids, eicosanoids, phospholipids.

The pairwise comparison of compounds that differentiated DHF/DSS from DF diagnosis groups yielded P-values that ranged from 1.63E-04 with a FC of -2.17 for a phenylacetylphenylalanine to 4.98E-03 with a FC of -1.59 for a phosphoinositol isotype.

The pairwise comparisons of compounds that differentiated DHF/DSS from ND diagnosis groups yielded P-values that ranged from 2.09E-07 and a FC of -2.23 for a phosphatidylinositol isotype to a P-value of 7.65E-04 and a FC of 4.91 for a potentially identified pantothenic acid. The pairwise comparisons of compounds that differentiated DF from ND diagnosis groups yielded P-values of 1.09E-02 with a FC of 2.31 for a compound potentially identified as of octadecadienoic acid to a P-value of 1.10E-05 and a FC of 5.26 for potentially identified deoxyadenosine monophosphate.

Considerable differences were found in saliva among Nicaragua and Mexico. This could be attributable to saliva being a very variable specimen, to differences in collection and handling of specimens, or to differences in diet in the two countries. The analysis of saliva specimens and comparison between Nicaraguan and Mexican samples suggested that there are limitations on the quality of saliva data.



	Purine	Hypoxanthine	Guanine	Xanthine	Uridine	Deoxyinosine	Inosine	Deoxyadenosine monophosphate	Serine	L-2-Aminoadipic acid	Methyl N-glycine	Citrulline	Glutamic acid-Leucine	Leucine-Glutamic acid-Histidine	Methionine-Phenylalanine-Histidine	Phenylacetylphenylalanine	3,6-octadecadiynoic acid	α-Linolenic Acid	9-10,12-octadecadienoic acid	Palmitic acid	Prostaglandin J2	Pantothenic Acid	Riboflavin	LysoPE(0:0/18:3)	PI(12:0/12:0)	PC(14:1/16:1)
DHF/DF	0	1.28	0	0	0	0	0	0	0	0	0	0	0	-1.48	0	-2.17	0	0	0	0	0	0	0	0	-1.59	0
DHF/ND	4.51	0	2.99	4.25	3.74	4.33	4.19	3.55	4.01	3.71	3.32	2.69	3.62	0	5.07	0	2.49	2.76	3.12	4.71	3.99	4.91	3.02	3.13	-2.23	4.11
DF/ND	2.81	0	3.19	3.97	4.32	4.41	4.09	5.26	0	3.41	4.29	3.27	4.38	0	2.49	1.84	2.81	3.2	2.31	4.81	3.02	4.25	2.77	2.55	0	3.39

Figure 29. Selected candidate compounds detected in Mexican saliva specimens by HILIC-LC-MS analysis that differentiate the DHF/DSS, DF, and ND diagnostic groups. Figure presents selected candidate compounds and whether they were increased or decreased in abundance in pairwise comparisons of different disease states. The color of each bar represents the respective pairwise comparison; blue bars represent DHF/DSS vs. DF, red bars represent DHF/DSS vs. ND, green bars represent DF vs. ND.

Table 28 . Selected candidate compounds detected in Mexican saliva specimens that differentiate the DHF/DSS, DF and ND diagnostic groups by HILIC LC-MS analysis*

Mexican saliva			Calculated formula	#DB hits	Detected in samples			DHF/DSS-DF		DHF/DSS-ND		DF-ND	
Mass	RT	Potential compound			% DHF	% DF	% ND	P value	FC	P value	FC	P value	FC
Nucleosides													
120.0436	1.38	Purine	C5H4N4	14	53	49	27	-	-	2.13E-03	4.51	3.21E-03	2.81
136.0386	7.15	Hypoxanthine	C5H4N4O	11	47	54	93	2.04E-03	1.28	-	-	-	-
151.0495	10.67	Guanine	C5H5N5O	5	53	49	87	-	-	4.83E-03	2.99	2.41E-03	3.19
152.0334	2.98	Xanthine	C5H4N4O2	4	93	100	97	-	-	3.64E-04	4.25	8.14E-04	3.97
244.0697	2.98	Uridine	C9H12N2O6	18	87	85	87	-	-	2.08E-03	3.74	5.27E-04	4.32
252.0861	6.48	Deoxyinosine	C10H12N4O4	18	53	36	20	-	-	1.19E-03	4.33	4.91E-04	4.41
268.0805	5.72	Inosine	C10H12N4O5	35	87	97	93	-	-	9.22E-04	4.19	2.82E-04	4.09
331.0671	3.13	Deoxyadenosine monophosphate	C10H14N5O6P	3	33	51	10	-	-	3.04E-20	3.55	1.10E-05	5.26
Amino acids, peptides and derivatives													
105.0429	2.13	Serine	C3H7NO3	3	67	41	27	-	-	8.15E-04	4.01		
161.0685	1.94	L-2-Aminoadipic acid	C6H11NO4	12	67	51	7	-	-	1.47E-03	3.71	4.85E-03	3.41
173.1044	1.42	Methyl N-(a-methylbutyryl)glycine	C8H15NO3	11	80	59	23	-	-	2.82E-03	3.32	1.27E-03	4.29
175.0957	18.76	Citrulline	C6H13N3O3	5	80	72	47	-	-	1.32E-02	2.69	4.35E-03	3.27
260.1367	2.28	Glutamic acid-Leucine	C11H20N2O5	11	60	41	20	-	-	5.92E-06	3.62	7.16E-04	4.38
397.1958	25.76	Leucine-Glutamic acid-Histidine	C11H20N2O5	21	20	26	67	1.33E-03	-1.48	-	-	-	-
433.1818	25.76	Methionine-Phenylalanine-Histidine	C20H27N5O4S1	6	53	49	47	-	-	1.10E-04	5.07	3.09E-06	2.49
283.117	6.80	Phenylacetylphenylalanine	C17H17NO3	9	33	33	63	1.63E-04	-2.17			2.28E-05	1.84
Fatty acids													
276.209	1.14	3,6-octadecadiynoic acid	C18H28O2	118	8	31	113	-	-	4.85E-03	2.49	8.85E-03	2.81
278.2241	1.15	α-Linolenic Acid	C18H30O2	82	8	28	107	-	-	3.10E-03	2.76	5.02E-03	3.20
296.2349	1.14	9-10,12-octadecadienoic acid	C18H32O3	57	47	64	30	-	-	5.54E-03	3.12	1.09E-02	2.31
256.2399	1.20	Palmitic acid	C16H32O2	43	20	31	60	-	-	4.81E-03	4.71	4.78E-04	4.81
Eicosanoids													
316.2036	1.16	Prostaglandin J2	C20H28O3	76	67	82	70	-	-	1.33E-03	3.99	3.85E-03	3.02
B Vitamins													
219.1105	1.88	Pantothenic Acid	C9H17NO5	6	93	90	80	-	-	5.24E-05	4.91	3.04E-04	4.25
376.1381	4.62	Riboflavin (Vitamin B2)	C17H20N4O6	22	67	77	73	-	-	4.53E-03	3.02	2.81E-03	2.77
Phospholipids													
475.2576	1.28	LysoPE(0:0/18:3(6Z,9Z,12Z))	C23H42NO7P	7	73	69	77	-	-	3.10E-04	3.13	9.95E-03	2.55
698.4025	2.61	PI(12:0/12:0)	C33H63O13P	1	40	38	77	4.98E-03	-1.59	2.09E-07	-2.23		
701.4929	1.41	PC(14:1(9Z)/16:1(9Z))	C38H72NO8P	23	73	36	17			3.64E-04	4.11	6.81E-03	3.39

* The table shows the mass, retention time (RT), calculated formula, a potential identity and the number of hits found in the database.

4.E. DISCUSSION.

A metabolomics LC-MS approach was optimized and used to determine metabolic profiles of acute phase serum, saliva, and urine samples from patients presenting with DEN-like diseases in Nicaragua and Mexico. The goal was to identify molecular features altered during DENV infections and associated with DEN disease outcome. The potential compounds corresponding to the respective molecular features were then to be identified *in silico* and their potential biological and physiological significance was to be ascertained. The dissertation discussion and analysis will be predominantly focused on the serum results; results with the saliva and urine specimens were more variable and should be considered more preliminary than the serum results. This issue will be discussed in more detail later.

Clearly DENV infection perturbs the metabolome of the infected individual, and many molecular features (Tables 17 - 20) and potential compounds (Tables 21 - 28) differentiated the diagnosis groups DHF/DSS, DF, and ND. This bodes well for downstream chemical identification of specific metabolites of DEN disease outcomes. In serum the majority of the molecular features and compounds identified *in silico* were present in the samples from both countries, but only molecular features that met the statistical criteria of $P \leq 0.05$, FC of ≥ 2 (FC could be positive or negative) and corrected P-value of ≤ 0.05 in serum, and a modified FC of >1 for non-invasive samples, were included in the Appendices. The appendices include the compounds listed in Tables 17 to 20, and each molecular feature is listed by its neutral mass, retention time, calculated formula and statistical values obtained in pairwise comparisons among DEN diagnosis groups.

The compounds obtained with RP analysis (Appendix 3) were not pursued in this dissertation. The biological relevance and identification of these could be pursued in further analysis.

It is certainly possible that other molecular features could have diagnostic and prognostic value under certain circumstances. This possibility will be investigated by collaborators at the University of California

– Berkeley. Nonetheless, several of the potential candidates that differentiated the diagnosis groups are provocative in terms of aiding our understanding of DENV pathogenesis and predicting disease outcomes. Some of these findings will be described briefly below with the caveat that many of the *in silico* detected compounds remain to be identified by LC-MS/MS. The identities of six metabolites in serum, two in urine and one in saliva have been confirmed by LC-MS/MS. These metabolites and their potential roles in DENV pathogenesis and disease outcome will be discussed in Chapter 5. A brief discussion of the *in silico* identified, potential candidate compounds perturbed by DENV infection follows.

DENV induced changes in amino acid and sugar-metabolism: The metabolomics analysis of human specimens revealed DENV infections appeared to induce perturbations of amino acid and sugar metabolism in serum, urine, and saliva (Tables 21, 23, 25 and 28). Examples of the potential altered amino acids detected include proline, valine, arginine, methionine, 2-aminoadipic acid among others. Previous metabolomic analyses by others have revealed carboxylic acid, amino acid, and sugar metabolites are commonly affected in different types of infectious diseases. It has been postulated that amino acids could be elevated as a part of the response of endothelial cells to oxidative stress. [283-285] This could be relevant for a theoretical role of the disturbed abundances of amino acids that differentiated the DEN diagnosis groups.

DENV infection was associated with altered levels of amino acid metabolites in serum, urine and saliva; this could indicate a perturbation of host cell metabolism. For example, serine was one of the candidates found to be elevated in urine and serum specimens from Mexican specimens by LC/MS (Tables 25, 27 and 28). Serine has been demonstrated to be increased in the medium of cell cultures infected with DENV- 3.[280] Serine functions in purine and pyrimidine metabolism, and the increase in its levels in DEN-specimens suggests interference in their catabolism induced by DENV infection.[281] Elevated levels of amino acids in serum can also be caused by proteolysis, proteolysis can occur in DENV

infection and other infections and is caused by cachexia or wasting syndrome characterized by fatigue, anorexia, weight loss, weakness secondary to the effects of DENV in the infected organism.

DENV- induced changes in phospholipid metabolism: Previous metabolomics studies with DENV investigated virus-induced modifications to the intracellular membranes of infected cells. [8, 56] These *in vitro* studies described important alterations in a specific phospholipid repertoire induced by DENV infection. Specifically these studies demonstrated that during DENV infection intracellular lipids are redistributed in the cell due an inhibition of the fatty acid synthase enzyme. Sphingolipids, phospholipids, and fatty acids all play important roles in DENV infection.[8] Importantly a recent metabolomics study of DEN in humans also revealed perturbation of the host lipidome. [7] These previous reports support the results found in this study demonstrating disruption of fatty acid, phospholipid and sphingolipid metabolism in mosquito cells and human hosts during DENV infection (Table 24).

It has been reported by others that in cell cultures infected with DENV-3 and DENV-4, metabolites of fatty acids were elevated. This suggests a possible interruption in metabolism of the saturated long-chain fatty acids via oxidation of medium chain fatty acids as a consequence of DENV infection perturbing amino acid and the tricarboxylic cycle (TCA) metabolism.[281] Fatty acids were found to be altered in the three types of specimens (serum, urine and saliva) examined (Tables 21-24, and 27 and 28).

Fatty acid biosynthesis and the enzyme fatty acid synthase have an important function in DENV replication in cultured human and mosquito cells.[8] It is certainly possible that DENV infection also perturbs these pathways in humans.[7] Fatty acid biosynthesis plays a vital role in virus replication. DENV-NS3 protein is responsible for redistribution of fatty acid synthase to cellular locations of viral replication and also increases cellular fatty acid synthesis. This would disrupt the metabolism of

infected cells,[8, 9, 281] causing the profiles of fatty acids and phospholipids to be altered during DENV infections, similar pathways as the ones reported may be disrupted in DENV infected humans. Perera et al, described the differences in the abundances of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelins (SM), ceramide, lysophospholipids (lysoPC) and ceramide phosphoethanolamine in DENV infected mosquito cells and postulated that these are due to alterations in cellular membrane architecture and signal transduction pathways. These lipids are relevant at different steps during viral replication; the steps affected include virus binding and entry. [8]

The findings presented in Table 24 support the published literature concerning the affected lipid classes during DENV infection. It is logical to hypothesize that similar function and pathways, e.g. membrane architecture and cellular signal transduction pathways, are perturbed in human cells.[7] Importantly in this regard, concentrations of some lysoPCs were found to be increased in serum samples of Nicaraguan and Mexican patients (Tables 21-28). LysoPCs are a product of the hydrolysis of phospholipids by the enzyme phospholipase A2, which results in a phospholipid with one acyl chain.

Because of the characteristics of its structure, the incorporation of lysoPC in membranes produces a curvature and induces vesicle fusion and budding of DENV. LysoPC also has a role in the permeability of the membranes and are considered proinflammatory and atherogenic, having the potential of increasing plasma membrane permeability and expression of proinflammatory molecules.[286-289] The transmembrane permeabilizing effect of lysoPC is augmented in the presence of palmitic acid, which has been found to be elevated in DENV infected cells [8] and human serum (Tables 21-23). This could have an important role in DEN severe cases. This will be discussed in greater detail in Chapter 5.

DENV induced changes in nucleoside metabolism: It has been shown that some nucleosides (e.g. inosine, uridine, and hypoxanthine) are elevated in the interstitial fluid during hypoxia and

reperfusion.[290] Inosine is thought to have strong immunomodulatory effects by enhancing mast-cell degranulation, it attenuates the production of pro-inflammatory mediators by macrophages, lymphocytes and neutrophils.[291]

Inosine was elevated in saliva and urine of DEN cases in our analysis; inosine is related to the endothelial glycocalix degradation in cases of cardiac ischemia and hypoxia. Inosine is released into extracellular space from cells upon metabolic stress, such as inflammation and ischemia, or from sympathetic nervous system stimulation as found in previous studies using metabolomics in DEN patients. And it has been postulated to have important immunomodulatory effects at the transcriptional level. [7, 290, 291]

Uridine is an endothelium-derived vasoconstrictive factor, which was found to be elevated in saliva of dengue cases in our analysis.[292] Cytosine is increased in patients with heart failure and with perturbations of the TCA cycle and fatty acid metabolism; it was found to elevated in urine of DEN patients in our analysis. Although no direct associations between DENV with nucleosides have been demonstrated, it is possible that some nucleosides could be elevated in endothelial damage during DHF/DSS. Further studies need to be done to corroborate this hypothesis.

Overall, the results of the LC-MS analysis were promising. These studies provided proof of principle that a metabolomics approach can be used to detect candidate compounds for DEN diagnosis and prognosis.

The results obtained in Mexican and Nicaraguan serum are especially intriguing because many of the candidate compounds detected coincide with those reported previously by Perera et al [8], and Cui et al [7]. Importantly, the expanding metabolic biosignatures of disease outcomes could also be exploited to further understand the pathogenesis of DENV in the human host and the pathogenic mechanisms resulting in the different disease outcomes.

Differences were found among statistically significant compounds among Nicaraguan and Mexican serum samples. This could be due to the differences in patients samples, including that the

Mexican samples were principally from adults (>90%) and only few were from children (<10%). In contrast, all of the Nicaraguan samples were from children. These differences should be addressed as variables in future studies, along with other epidemiological variable such as gender, diet, presence/absence of signs and symptoms of the disease, the infecting DENV serotype, differences in specimen collection times patients and other associated pathologies that could be present and alter the metabolome, such as obesity, anemia, other seasonal infectious diseases, among others.

The results with urine and saliva samples were much more variable. The LC-MS analysis of urine and saliva specimens from both countries revealed perturbations in candidate compounds from similar biochemical groups as detected in serum and among countries. However, the compounds that statistically differentiated disease outcomes differed in specimens from the two countries. No compounds that were statistically significant were found in common among Nicaraguan and Mexican saliva samples. The FC values for candidate compounds detected in Nicaraguan urine and saliva samples (Tables 25 and 27) were quite low and predominantly negative as compared to those detected with Mexican urine and saliva samples (Tables 26 and 28). The reasons for this remain to be determined.

Additional saliva and urine samples have been collected in Nicaragua and will be analyzed by LC-MS, which will reveal whether or not the previous samples had been compromised in some way. The raw data derived from the LC-MS analyses of urine and saliva from Mexico should be further analyzed to identify additional potential compounds that differentiate the disease outcomes. The fold changes obtained with these specimens suggests that these urine and saliva specimens have not been compromised prior to analysis.

In the case of saliva samples, which could be a preferred specimen for DEN diagnosis because of the ease of collection, the use of salivation inducing devices and saliva collection kits could provide samples that show less variability. Development of noninvasive diagnostic capability remains a major goal of the overall research program. Unfortunately, there are virtually no publications on the use of

metabolomics to identify SMBs of disease progression in urine and saliva of DEN patients. This would clearly be a fruitful area of research.

These studies have identified a number of potential compounds that could comprise or be a part of a metabolic biosignature of DEN disease and outcomes. It is important to note that all of the candidate compounds associated with particular diagnosis groups detected in serum, urine and saliva were identified by statistical analysis and *in silico,* and the potential identity of a compound was obtained by interrogating one of the human metabolite databases. In order to unequivocally identity a candidate compound LC-MS/MS is required to provide the spectrum of the metabolite and to compare that with a known standard. This process is the subject of and explained in detail in Chapter 5. Very importantly, the metabolic biosignatures of disease outcomes in serum can also be exploited to further understand the pathogenesis of DENV in the human host and the pathogenic mechanisms resulting in the different disease outcomes

CHAPTER 5

METABOLOMICS-BASED IDENTIFICATION OF SMALL MOLECULAR BIOMARKER MEABOLITES IN SERUM AND NON-INVASIVE CLINICAL SPECIMENS (URINE AND SALIVA) FOR DIAGNOSIS AND PROGNOSIS OF DENV INFECTIONS

5.A. INTRODUCTION

Metabolomics has emerged as a platform that differentiates and identifies metabolites that are directly attributable to metabolic changes secondary to infections and that differentiate disease states by revealing host responses to the pathogen, including tissue damage, inflammation, and other virus induced pathology. Metabolites are the products or intermediaries of metabolism of cells that could be affected by stress or infection; these can serve as valuable biomarkers for disease.[284]

Comparison of the metabolic profiles of diseased with healthy individuals can be used to identify metabolites that differentiate disease outcomes. Metabolomics has revealed major differences in lipid metabolic profiles of cultured cells and humans infected with DENV.[7, 8]This would be expected because DENV relies on the host cell to fulfill lipid requirements for viral replication. Some intracellular membranes are reorganized and proliferate during replication. Membrane-enclosed compartments are thought to help the virus to avoid the antiviral defense mechanisms of mammalian and insect cells.[9, 293] Identification of DENV-specific metabolites or perturbation of specific metabolic pathways could lead to development of a new generation of diagnostic and prognostic assays for DEN disease.

Metabolic disturbances in DENV-infected cells can be caused by replication of the virus and its requirements for host cell resources; these would alter the homeostasis of the cells. In addition induction of the host immune response to DENV infection would also perturb the host metabolome.

At a cellular level, clearly there are several factors induced by DENV that could cause alterations in the infected cell homeostasis. A brief summary of some of the effects caused by the virus and the

virus requirements for host cells resources that could be potentially responsible for the metabolic changes observed follow:

Non-structural protein 1 (NS1) plays an essential role in viral RNA replication. NS1 is expressed on the surface of and secreted from infected cells; this excreted NS1 can bind to the surface of infected and uninfected cells. In DENV secondary infections, a memory response of IgM and IgG antibodies to NS1 during the acute phase of the disease could cause the formation of immune complexes that can trigger inflammatory processes. For example, the activation of complement, which can cause endothelial cell retraction and increase vascular permeability, could cause the severe symptoms found in DHF/DSS.[55] Some NS1 antibodies are cross-reactive with human platelets and endothelial cells and may contribute to the severe disease manifestations; however the mechanisms of activation of DEN pathogenicity remain to be fully determined.[38]

The NS3 protein is responsible for fatty acid synthase (FASN) recruitment; FASN is essential enzyme of the host cells, which has a key role for the biosynthesis of fatty acids. In infected cells, NS3 protein can relocate and modulate FASN and with this is able to induce de novo lipid biosynthesis.[9] This is relevant to the results presented in this chapter because lipids are signaling mediators of regulatory events in the cell. Viruses as parasites rely on the host to fulfill their lipid requirements, which are essential for replication of DENV; the host derived membranes are rearranged to provide platforms for DENV replication factories.[57] In this regard, two fatty acids and two phospholipids were found to be statistically relevant in differentiating DEN disease states.

E protein (envelope) induces the predominant antibody response during DENV infection for being the major protein exposed on the virus surface; this protein binds cellular receptors and fuses with host cell membranes, eliciting immune responses. Neutralizing antibodies directed against the E protein are the principal mediators of protection against DENV infection; some E antibodies are cross-reactive with plasminogen, which could increase the manifestations of severe DEN infection. [38]

The DENV proteins mentioned in previous paragraphs are not the only possible causes of changes in metabolism in cells induced by infection; clearly there are several other factors involved. Host factors play a major role in the immunopathogenesis of DENV infections. The host immune response is activated by DENV infection, which can activate cytokines that could induce capillary leakage through direct and indirect effects in the vascular endothelium.[103] Pro-inflammatory components of the immune system could increase the damage to endothelial cells. Vascular leakage is caused by a diffuse increase in capillary permeability, which allows extravasation of fluid, thus favoring a capillary leak syndrome, which can progress to DSS.[103, 104]

The *in vivo* mechanism by which ADE causes DHF/DSS remains unclear. Non-neutralizing antibodies facilitate virus entry into host cells. The non-neutralizing antibodies that bind to E or prM and to FcγR can enhance infection of the FcγR bearing cells. [142] Increasing the amount of DENV-infected circulating cells and induces a more intense immune response. Greater viral replication and immune activation would promote greater cytokine release, inflammation, tissue damage, and hemorrhagic manifestations, and vascular leakage.

Overall, both viral factors and host factors could predispose a patient to present a mild case of DEN or to present the most severe forms of the disease, DHF/DSS; metabolic changes in human cells caused by DENV infection and the host immune response can be subjected to metabolomics analysis for detection and profiling.

A critical step in the metabolomics approach is the unequivocal identification of the compounds that represent the SMBs or metabolites that are associated with different disease states detected by LC-MS. Tandem-mass spectrometry analyses (LC-MS/MS) provide the platform to detect and identify SMBs that can be correlated with disease prognosis in acute samples. The mass spectrum resulting from the tandem LC-MS fragmentation can be compared with that of a known standard or a mass spectrum library to determine the chemical identity of the metabolite

In this chapter, selected *in silico*-identified compounds that differentiate DHF/DSS, DF and ND disease in Mexican and Nicaraguan serum, urine, and saliva samples that were presented in Chapter 4, were further characterized using LC-MS/MS to confirm the chemical identity of the candidate SMBs. The spectrum of the compound in the native serum, urine, or saliva specimen was compared with that of a commercially available standard or in the METLIN online database or in the National Institute of Standards and Technology (NIST) mass spectrum libraries.

The LC-MS/MS analyses identified a panel of candidate SMBs, including phospholipids, fatty acids, a vitamin D isotype, nucleosides and amino acids and derivatives, in acute phase serum, saliva, and urine of DEN patients for potential use in prognosis and diagnosis of DEN infections using a metabolomics approach.

5. B. STUDY DESIGN, APPROACH, AND WORKFLOW

A subset of the selected *in silico* identified compounds from LC-MS analysis (Chapter 4) were further analyzed with tandem mass spectrometry (LC-MS/MS) to corroborate the identity of the actual compounds involved. Acute phase serum, urine, and saliva specimens from DENV-infected and non-dengue (ND) patients from Nicaragua and Mexico were subjected to a targeted LC-MS/MS to confirm (or not), the chemical identity of the respective *in silico* identified compounds.

A mass spectrum can help identify a compound by comparison of spectra of the feature with the spectra of a known standard. The identification of SMBs of native samples by comparison with a commercially available standard is a common strategy to confirm or determine the chemical identity of a metabolite. When available, commercial standards were purchased and the MS/MS spectrum of the standard and the SMB in the native sample were compared. If a commercial standard was not available for the *in silico*-identified compound, the spectrum obtained from LC-MS/MS analysis of the native specimen was compared to spectrum available on line or in public or commercial libraries.

The protocols for sample preparation prior to LC-MS/MS analyses were consistent with the protocols used for the untargeted part of the analysis performed with LC-MS (Chapter 4). Prepared samples were subjected to LC-MS/MS; the *m/z* (mass to charge) of the selected compound and its MS/MS spectrum was then compared with the MS/MS spectrum of a purchased standard (when available) or to spectrum available on the METLIN online database or the NIST mass spectrum library.

METLIN is an open source library that has been developed to comprehensively and automatically search LC-MS/MS data against high quality experimental MS/MS data from known metabolites. The NIST library is a commercial collection of MS/MS spectra that allows searching in mass spectrum libraries for exact mass of a precursor ion using its exact *m/z*.

5. B.1. Material and Methods. The materials and methods used for the targeted phase of this profiling study are similar to the materials and methods used in the untargeted phase of the study.

5.B.1.a. Clinical samples. Selected clinical acute-phase from febrile patients included serum and linked serum, urine and saliva, which were previously analyzed by LC-MS (Chapter 4), were analyzed by tandem-mass spectrometry (LC-MS/MS). Briefly, these specimens were collected from patients presenting with DEN-like disease in Nicaragua and Mexico and provided to CSU by collaborators. The specimens had no personal identifiers but information on the final diagnosis of the case (DF, DHF/DSS, and ND) was provided.

5.B.1.b. Processing of clinical specimens for LC-MS/MS analysis. The protocol used for serum, urine and saliva sample preparation for LC-MS/MS was the same as described in Chapter 4 (4.B.3) for LC-MS analysis.

5.B.2. Commercial standards for LC-MS/MS analysis. For the targeted LC-MS/MS studies for compound identification, standards that were commercially available were purchased. Mass spectra of the standards and of the SMBs in the native serum were compared. A total of 17 standards were purchased and compared with SMBs potentially identified *in silico* in serum specimens (Table 29). Every standard

was diluted in acetonitrile (ACN) to a concentration of 1 µg/ml. The standards were then pooled and injected directly to the LC-MS/MS.

Table 29. Commercial standards to identify candidate SMBs in serum*

Commercial names of standards purchased to compare to serum	Chemical formula	Monoisotopic Mass	*m/z*	RT (min)
Amino acid				
Proline	C5H9NO2	115.0633	116.0633	14.2
Fatty acids				
α-linolenic acid	C18H30 O2	278.2246	279.2246	1.10
Docosahexaenoic acid	C22 H32 O2	328.2402	329.2402	1.09
5,6-dehydro arachidonic acid	C20 H30 O2	302.2245	303.2245	1.13
9,12-octadecadiynoic acid	C18 H28 O2	276.2089	277.2089	1.11
Phospholipids				
1-oleoyl-lysophsphatidylcholine	C26 H52 N O7P	521.3481	522.3481	13.74
Platelet activating Factor C-16	C26 H54 NO7 P	523.3637	524.3637	14.72
1-palmitoyl-lysophosphatidyl-choline	C24 H50 NO7 P	495.3324	496.3324	14.9
Vitamin D isotypes				
1α,25, dihydroxyvitamin D3	C27 H44 O3	416.3290	417.3290	1.3
Vitamin D3	C27 H44 O	384.3387	385.3487	1.14
25-hydroxy-vitamin D3	C27 H44 O2	400.3312	401.3412	1.25
Eicosanoids				
Prostaglandin I3	C20 H29 O5 Na	372.1912	373.1912	1.21
Hydroxy prostaglandin F2	C20 H34 O6	370.2355	371.2355	1.21
Thromboxane B2	C20 H34 O6	370.2355	371.2355	1.21
Prostaglandin E3	C20 H30 O5	350.2093	351.2093	1.10
Prostaglandin D3	C20 H30 O5	350.2093	351.2093	1.10
Eicosapentaenoic acid	C20 H30 O 2	302.2243	303.2343	1.13

*Table shows the identity, chemical formula, monoisotopic mass, m/z and retention time of the commercial standards. The compounds with identical mass, chemical formula and RT differ only by their structural formula.

A total of 6 standards were purchased and compared with the mass spectra of the compounds found in non-invasive samples in order to confirm the identity of potential candidate compounds (Tables 30 and 31). As with serum, every standard was diluted in acetonitrile (ACN) to a concentration of 1 µg/ml. The standards were then pooled and injected directly to the LC-MS/MS.

Table 30 . Commercial standards to identify candidate SMBs in urine*

Commercial name of standards purchased to test in urine	Chemical formula	Monoisotopic mass	*m/z*	RT
Amino acid				
Arginine	C6 H14 N4 O2	174.1117	175.1217	25.85
Nucleosides				
Cytosine	C4 H5 N3 O	111.0435	112.0535	13.228
2'Deoxyinosine	C10 H12 N4 O4	252.0858	253.0958	3.40
Inosine	C10 H12 N4 05	268.0811	269.0911	5.37

*Table shows the identity chemical formula, monoisotopic mass, m/z and retention time of the standards.

Table 31 . Commercial standards to identify candidate SMBs in saliva*

Standards purchased to test in saliva	Chemical formula	Monoisotopic mass	*m/z*	RT
Amino acids and derivatives				
L-citrulline	C6 H13 N3 O3	175.0957	176.1057	18.76
Serine	C3 H7 N O3	105.0429	106.0529	2.13
Nucleosides				
2'Deoxyinosine	C10 H12 N4 O4	252.0858	253.0958	3.40
Inosine	C10 H12 N4 05	268.0811	269.0911	5.37

*Table shows the identity chemical formula, monoisotopic mass, m/z and retention time of the standards.

5.C. LIQUID CHROMATOGRAPHY-TANDEM-MASS SPECTROMETRY ANALYSIS

5.C.1. Liquid chromatography (LC). A hydrophilic interaction chromatography (HILIC) column was coupled to an Agilent 1200 series high-performance liquid chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA) for the analysis, as previously described in Chapter 4 (4.B.4.).

The LC-MS/MS analysis conditions, including solvents and gradient, were the same as those used for LC-MS analysis in Chapter 4. The samples were dissolved in a non-polar solvent, typically 95% acetonitrile (B), and loaded onto the column. The mobile phase was gradually changed to increase water content (A) stepwise (Table 32).

Table 32 . Tandem liquid chromatography gradient*

Time	Gradient
0.2 – 30 minutes	95% B to 50% B
30 – 35 minutes	50% B
35 – 40 minutes	50% B to 20% B
40 – 45 minutes	20% B to 95% B

*Non-linear elution gradient used in liquid chromatograph. The gradient is expressed as percentage of solvents (acetonitrile (B) to water) changes at different time points during LC-MS/MS analysis. Water accounts for the missing percentage.

5.C.2. Tandem mass spectrometry (MS/MS). LC-MS/MS was performed using the native samples and the selected commercial standards in a targeted fashion. Briefly, a list of the masses and retention times of the candidate compounds was annotated for targeted fragmentation of each compound (identified by mass and retention time) and detection in the LC-MS/MS.

The eluent from LC was introduced into the Agilent 6520 Quadrupole time of flight (Q-TOF) mass spectrometer coupled with dual electrospray ionization. LC-MS/MS analyses were performed using the same solvents as mentioned for LC and the same reference ions (121.0508 *m/z* and 922.0097 *m/z*). The data were collected in positive mode (in centroid and profile mode in 4 GHz high resolution mode). The parameters used are provided in Table 33.

Table 33 . Time of flight tandem-mass spectrometry parameter values for detection of *m/z* of SMBs from prepared samples*

Acquisition Method for tandem mass spectrometry	
MS/MS range	100-1700 Da
MS/MS scan rate	1.41 seconds
Ramped collision energy	
Slope	6.5
Offset	2
Source Parameters	
Gas Temp	325°C
Gas Flow (l/min)	10
Nebulizer	45 psig
Ion polarity	Positive
Scan source parameters	

Vaporizer capillary	4000 Volts
Fragmentor	150 Volts
Skimmer	65 Volts
OctopoleRFPeak	750 Volts
Binary Pump	
Collection time	45 minutes
Flow	0.4 ml/min
Pressure	400 bar
Solvent A	Water, 0.1% formic acid
Solvent B	Acetonitrile, 0.1% formic acid

*Values of parameters used for tandem-mass spectrometry data acquisition.

5.D. DATA PROCESSING AND ANALYSIS

5.D.1. LC-MS/MS chemical identification of SMBs using commercial standards. The spectrum obtained from the targeted analysis of each candidate SMB in the native sample was compared to the spectrum of a commercial standard when available using Mass Hunter Qualitative Analysis version B.05. The spectra were visualized using the "MS spectrum results" tool and the peaks resulting from the precursor ion (the *m/z* of the candidate compound fragments) were compared to the "daughter" peaks obtained from the fragmentation of the commercial standard ion. When a commercial standard was not available the spectrum was visualized using the tool mentioned above and compared to online MS/MS data available through METLIN and NIST. For being this a qualitative analysis, the relative abundances of each peak were not compared for MS/MS identification purposes.

5.D.2. LC-MS/MS identification of SMBs using the METLIN online data base. For METLIN identification, the peaks obtained from the fragmentation with LC-MS/MS were imported into the website http://metlin.scripps.edu/. Briefly, in the home page, the "search" command provided a drop window that contains the option "MS/MS spectrum match". Once selected, the *m/z* and intensity of a maximum of 30 peaks per compound were entered; these peaks could be imported or copied from the list of peaks of the fragmented candidate compound precursor ion from LC-MS/MS analysis visualized using the "MS spectrum results" tool in Masshunter.

The mode selected for this search was “positive” and the adduct selected was M+H only. The collision energy varied for each compound. The tolerance used for MS/MS was 0.01 Da, the maximum tolerance allowed for the precursor ion was 20 ppm (default of the program; it can be modified). The *m/z* of the precursor ion of the compound being analyzed was required for the analysis. The results were displayed in a new window.

5.D.3. LC-MS/MS identification of compounds using the NIST data base. For NIST-based identification, the *m/z* of the precursor ion of interest and its fragmented ions were imported into the NIST MS/MS library database (access kindly provided by the Proteomics and Metabolomics facility, CSU). The *m/z* of the precursor ion, its mass, retention time, and calculated formula were searched in the library of mass spectra and matched to a known and identified MS/MS spectrum. The NIST search provided the best match for the candidate SMB identified, and also provided matches that had a MS/MS spectrum similar to the potentially identified SMB. The parameters used to corroborate that the MS/MS spectrum was accurate included a dot-product value >600 and a reverse-dot product value >600. The largest dot-product and reverse-dot possible is 999.

The dot product mass spectrum search algorithm scales peaks using the square root of their abundance. For the identity search, the peaks were weighted by the square of their *m/z* value and a second term was added that compared ratios of adjacent peaks in library and unknown spectra. The reverse-dot match value is obtained by the program ignoring all the peaks present in the sample spectrum that are not in the library spectrum, meaning it only match the peaks “known” to the library.

Briefly, the four levels of identification accuracy for MSI are: 1. Identified compounds (by analyzing an authentic compound under identical experimental conditions and comparing to the candidate compound). 2. Putatively annotated compounds (without reference standards based on spectrum similarity with public/commercial spectrum libraries). 3. Putatively characterized compound classes. 4. Unknown compound. Each compound was assigned a level accuracy after identification.

5.E. RESULTS – Identification of candidate SMBs in native serum, urine and saliva samples.

The mass spectrum of each LC-MS/MS compound from native samples was compared to the mass spectrum of commercial standards or MS/MS libraries such as METLIN and HMDB. The comparison of spectra from native samples and spectra from commercial standards was performed manually and based on the similarities of the *m/z* of the peaks visible in the spectrum.

The most abundant peaks present in the tandem spectrum of each native sample and the respective commercial standard being compared were obtained from Masshunter Qualitative analysis; the peaks were then compared among the two spectra (commercial standard and native sample) and matched. For compounds lacking standards, an automatic comparison with online libraries or database libraries was performed as described above (5.D.2 and 5.D.3).

The comparison of the mass spectrum of a candidate compound in in serum, urine, or saliva specimens with either a commercial standard or a library allowed the identification and identification of six metabolites in serum, potentially two in urine and one in saliva (Table 34).

Table 34 provides the neutral mass, the retention time, the identity of the SMB, the chemical formula, the approach by which it was identified (commercial standard, METLIN or NIST and LC-MS/MS), statistical values, and the minimum reporting standards (MSI) for chemical analysis and statistical values for the pairwise comparisons of the compounds by diagnosis groups. The SMBs reported have MSI levels of 1 and 2. The biochemical classes of these candidate SMBs include phospholipids, vitamin D derivatives, nucleosides, amino acids, nucleosides and fatty acids (Table 34).

In serum, 1-oleoyl-lysophatydylcholine, 1-palmitoyl-lysophosphatydilcholine, α-linolenic acid, docohexaenoic acid and 1,25-dihydroxyvitamin D3 were identified. In urine, arginine and deoxyinosine were potentially identified using NIST and METLIN libraries respectively, and in saliva xanthine was identified using the NIST library

The comparison of LC-MS/MS spectra of commercially available standards with native urine and saliva samples (Table 34) did not result in identification of any candidate SMBs. For that reason, the candidate metabolites presented in Table 34 regarding urine and saliva specimens were identified by comparison of spectra of the native specimens with those provided in libraries as mentioned above; therefore metabolites reported were at level 2 of MSI standard of identification. The candidate metabolites were nucleosides and amino acids.

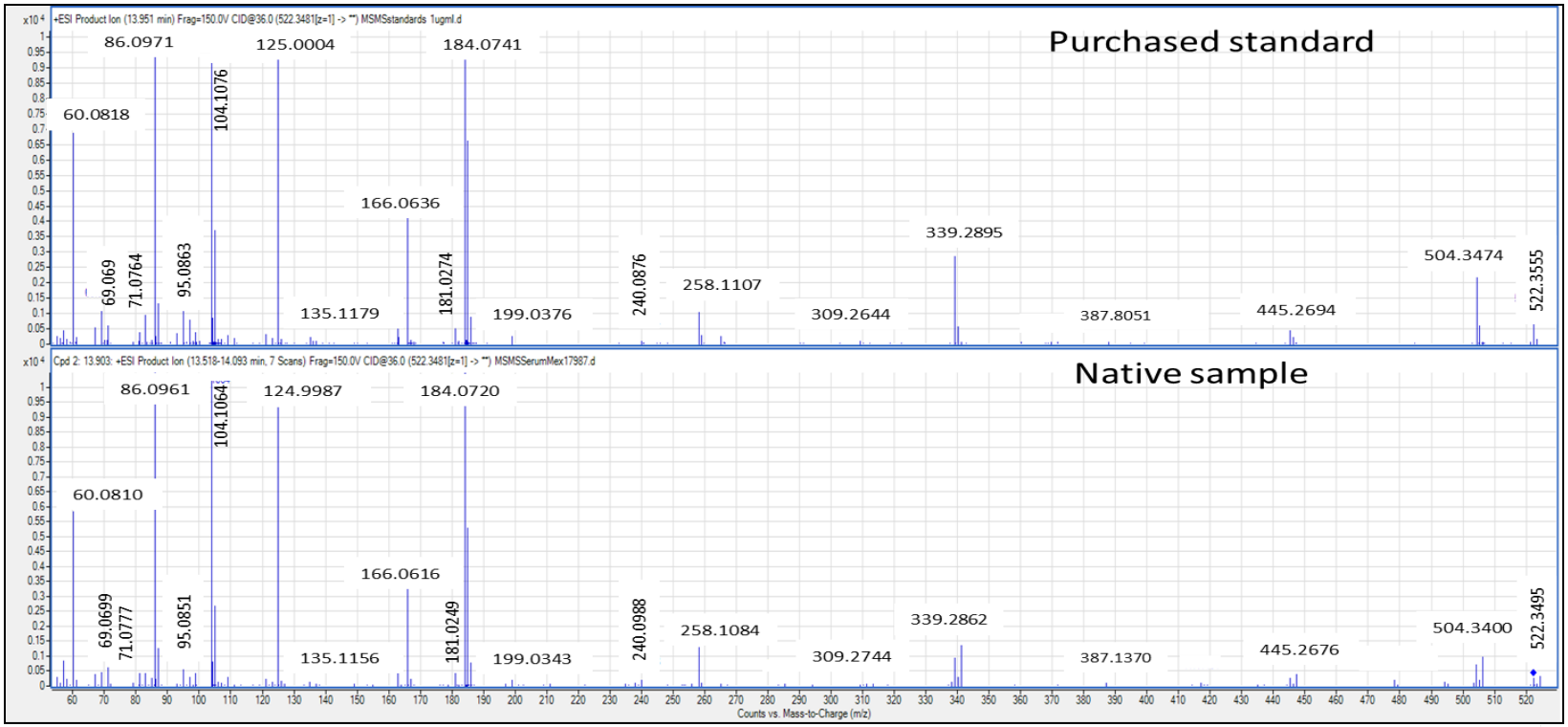
From the identified compounds, 5 compounds are able to differentiate DHF/DSS from DF in serum of Mexico or Nicaragua. In urine, arginine can differentiate DHF/DSS from DF and ND patients, and xanthine in saliva specimens, can differentiate DHF and DF from ND Mexican patients.

The results obtained indicate that the response caused by DENV infection in the host can be detected at a metabolic level due to disturbances in cellular metabolism and homeostasis. The candidate metabolites identified thus far by LC-MS/MS include lysophospholipids, fatty acids, one amino acid and a vitamin D3 isotype in serum and nucleosides, amino acids, and purines in urine and saliva.

Table 34 . LC-MS/MS identification of candidate metabolites for DEN diagnosis and prognosis of severe disease in clinical specimens*

				NICARAGUA						MEXICO							
				DHF/DSS-DF		DHF/DSS-ND		DF-ND		DHF/DSS-DF		DHF/DSS- ND		DF-ND			
Serum																	
Mass	**RT**	**Metabolite**	**Chemical Formula**	**P val**	**FC**	**P val**	**FC**	**P val**	**FC**	**P val**	**FC**	**P val**	**FC**	**P val**	**FC**	**Identified by**	**MSI**
Phospholipids																	
521.3481	13.90	1-oleoyl-lysophosphatidylcholine	C26 H52 N O7P	1.02E-03	9.16	4.03E-04	9.50	.	.	.	.	1.24E-03	360	2.47E-04	8.49	Standard +LC-MS/MS	1
495.3324	14.97	1-palmitoyl-lysophosphatidyl-choline	C24 H50 NO7 P	9.29E-03	7.55	4.97E-03	7.95	.	.	.	.	1.22E-03	9.60	2.42E-04	10.07	Standard + LC-S/MS	1
Fatty acids																	
278.2245	1.10	α-Linolenic acid	C18H30 O2	1.20E-06	10.94	7.82E-06	10.01	.	.	.	.	.	.	.	.	Standard + LC-MS/MS	1
328.2402	1.15	Docosahexaenoic acid	C22 H32 O2	1.98E-04	7.44	8.39E-05	7.61	.	.	.	.	.	.	.	.	Standard +LC-MS/MS	1
Vitamin D																	
416.3363	1.31	1,25-dihydroxy vitamin D3	C27 H44 O3	5.33E-03	-5.03	7.35E-03	-4.96	.	.	9.14E-01	-1	3.06E-04	-8.30	2.52E-04	-8.08	METLIN MS/MS spectrum database +LC-MS/MS	2
Amino acids																	
115.0635	14.44	Proline	C5H9NO2	2.26E-01	-2.15	6.65E-05	-8.88	5.37E-03	-6.74	.	.	.	.	.	.	Standard + LC-MS/MS	1
Urine																	
174.1116	23.4	Arginine	C6H14N4O2	.	.	.	.	.	.	3.68E-05	10.12	3.49E-05	9.57	.	.	NIST database +LC-MS/MS	2
Nucleoside																	
252.0858	3.41	Deoxyinosine	C10 H12 N4 O4	.	.	.	.	.	.	.	.	1.00E-07	2.12	.	.	METLIN MS/MS spectrum database +LC-MS/MS	2
Saliva																	
152.0334	2.99	Xanthine	C5H4N4O2	.	.	.	.	.	.	.	.	3.64E-04	4.25	8.14E-04	3.97	NIST database +LC-MS/MS	2

*The table shows the mass, retention time, candidate metabolite, chemical formula, the strategy used for identification and the Metabolomics Standards Initiative (MSI) grade of identification (1-4) and fold change (FC) in serum.



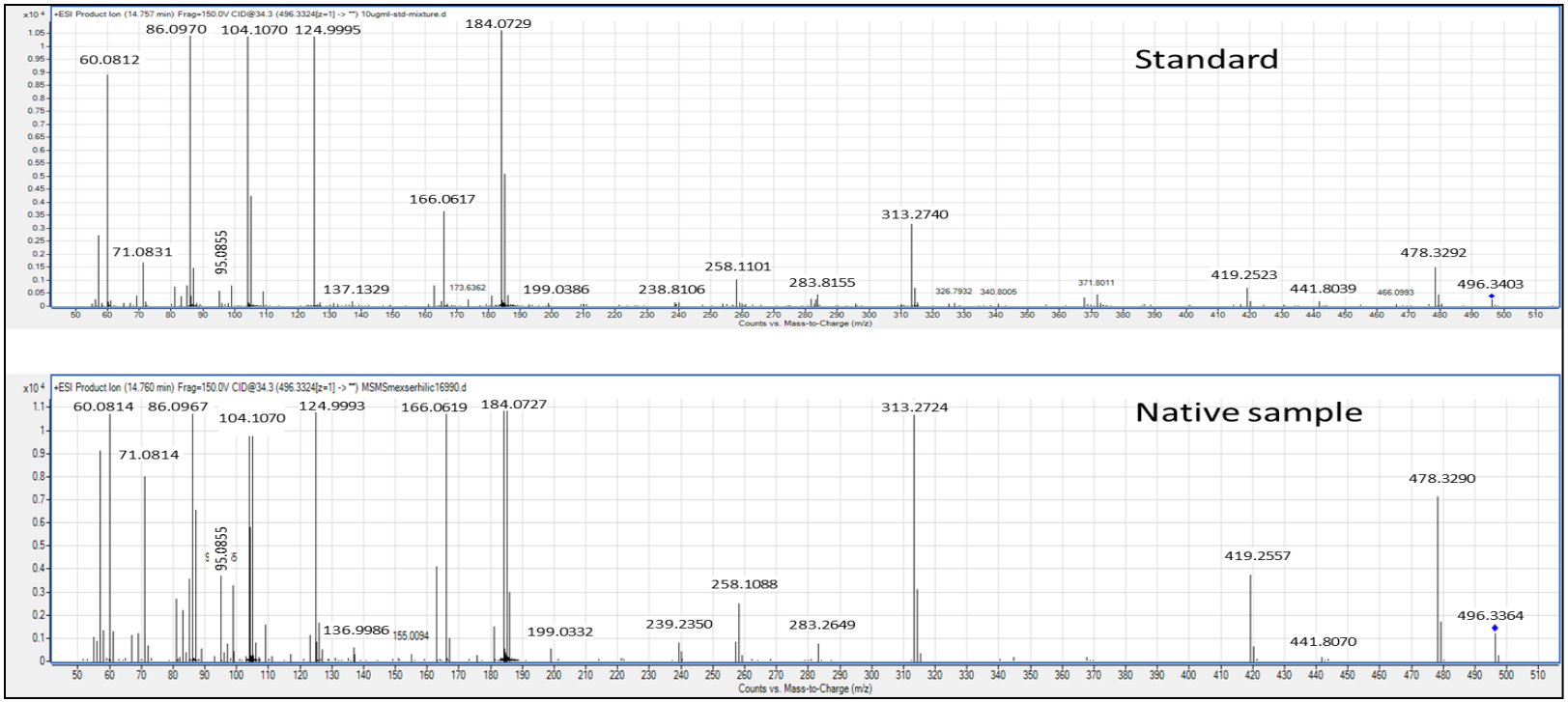
Standard

m/z	60.0818	69.0697	71.0764	86.0971	87.0996	104.1076	125.0004	135.1179	166.0636	181.0274	184.0741	185.0769	258.1107	339.2895	504.3474	522.3555

Serum

m/z	60.0801	69.0666	71.0752	86.0944	87.0977	104.1034	124.9971	135.1142	166.0575	181.0167	184.0687	185.071	258.1086	339.2792	504.5708	522.0623

Figure 30. Comparison of spectra of 1-oleoyl-lysophosphatidylcholine from a commercially available standard and a native serum sample. The mass spectrum of 1-oleoyl-lysophsphatidylcholine (top) was used to identify the native (bottom) compound by a manual comparison of the *m/z* values of selected peaks of a commercial standard and native sample respectively. The presence of peak 184.07 indicates that the compound contains a phosphatidylcholine (PC). The single fatty acid coupled to this PC is oleic acid, making this a lysoPC. Some of the peaks are not present in both spectra.



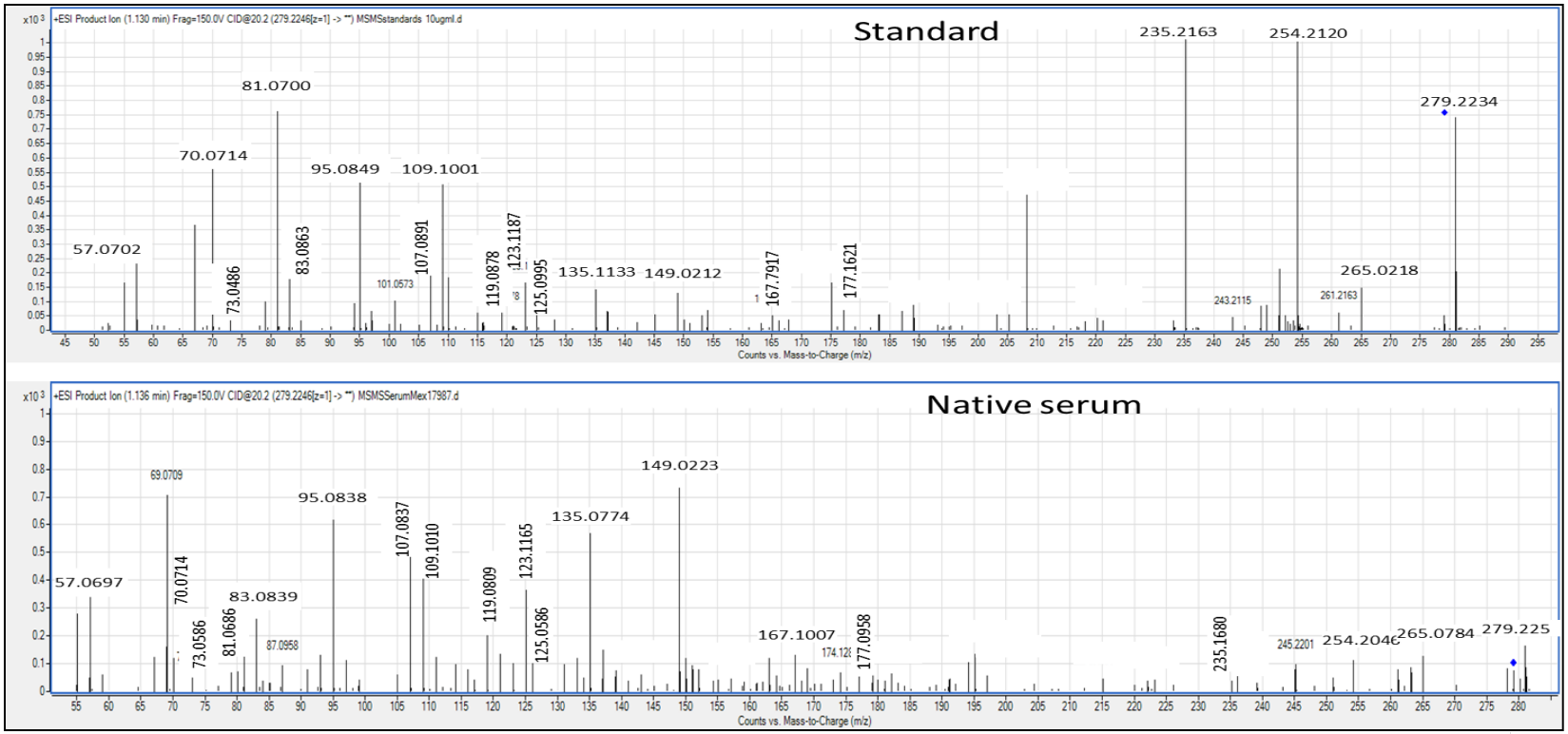
Standard

m/z	57.0708	60.0812	86.097	104.107	105.1107	124.9995	166.0617	184.0729	184.1971	185.0759	258.1101	313.274	419.2523	441.8039	478.3292	496.3403

Serum

m/z	57.0704	60.0814	86.0967	104.107	105.1098	124.9993	166.0619	184.0727	184.197	185.0758	258.1088	313.2724	419.2557	441.807	478.329	496.3364

Figure 31. Comparison of spectra of 1-palmitoyl-lysophosphatidylcholine from a commercially available standard and a native serum sample. The mass spectrum of 1-palmitoyl-lysophosphatidylcholine (top) was used to identify the native (bottom) compound by a manual comparison of the m/z values of selected peaks of a commercial standard and native sample respectively. The presence of peak 184.07 indicates that the compound contains a phosphatidylcholine (PC). The single fatty acid coupled to this PC is palmitic acid, making this a lysoPC. Some of the peaks are not present in both spectra.



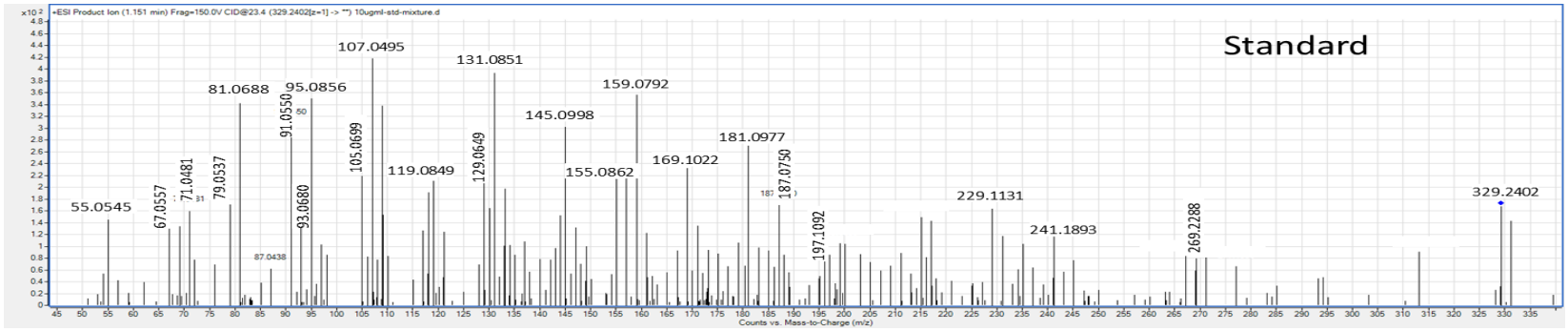
Standard

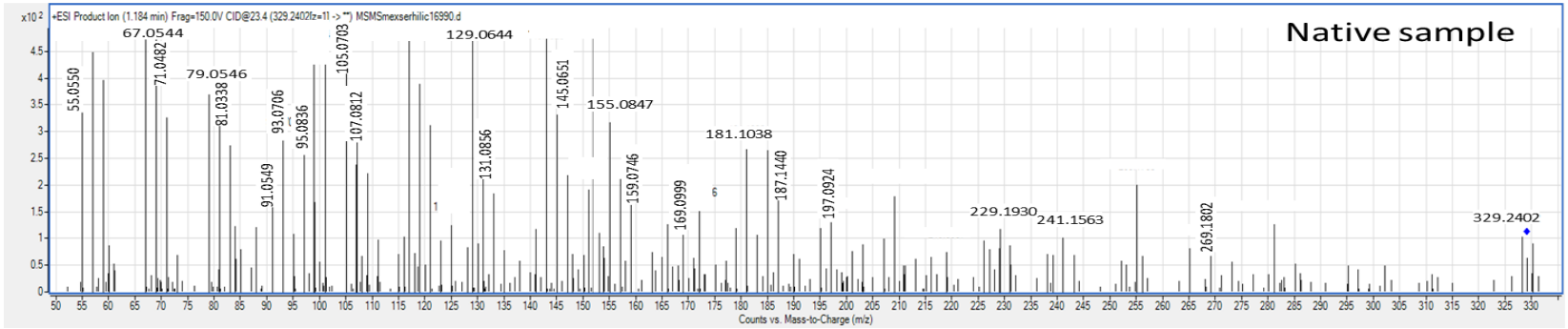
m/z	57.0702	70.0662	73.0486	95.0849	107.0891	109.1001	119.0878	135.1133	149.0212	167.7917	177.1621	208.2043	235.2163	254.212	265.0218	281.0479

Serum

m/z	57.0697	70.0714	73.0586	95.0838	107.0837	109.101	119.0809	135.0774	149.0223	167.1007	177.0958	208.157	235.2339	254.2046	265.0784	281.0413

Figure 32. Comparison of spectra of alpha-linolenic acid from a commercially available standard and a native serum. The mass spectrum of the α-Linolenic acid standard (top) was used to identify the native serum sample (bottom) by a manual comparison of the m/z values of selected peaks of a commercial standard and a native sample respectively. Some of the peaks are not present in both spectra.





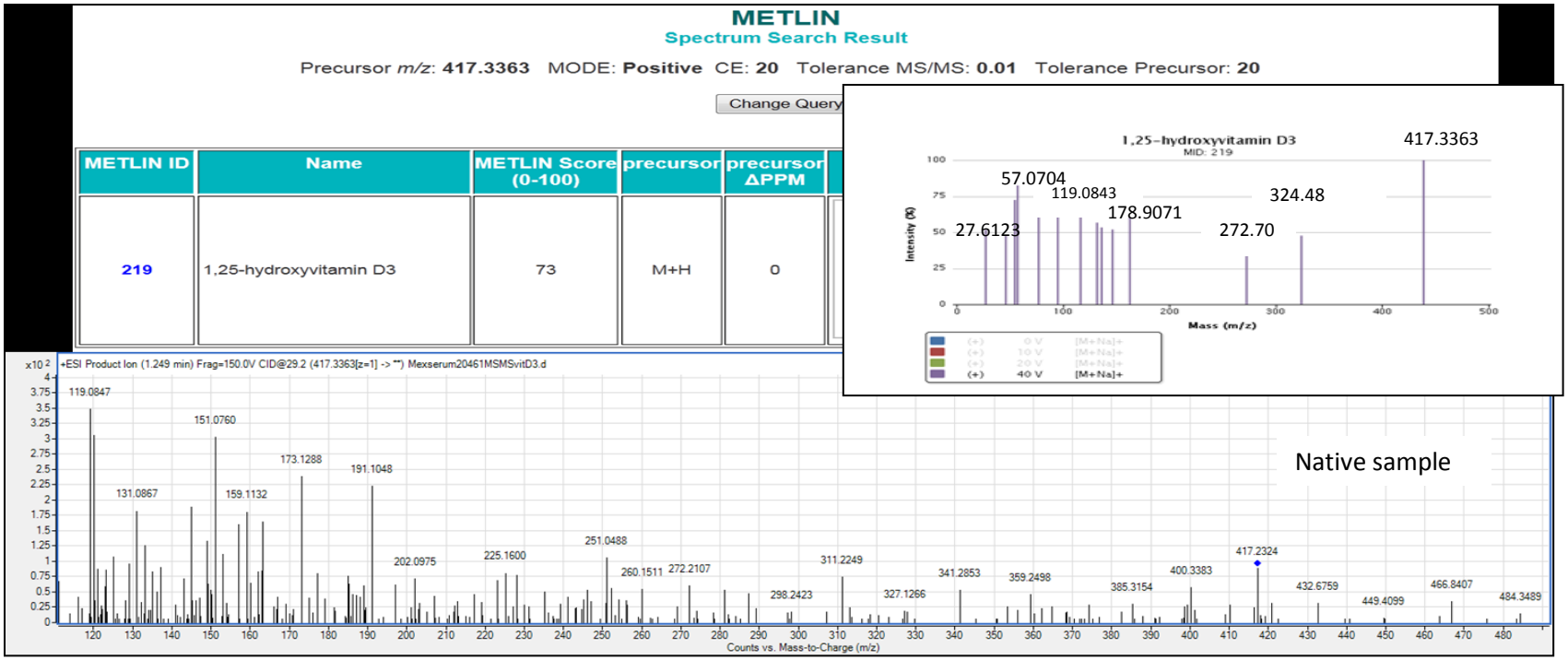
Standard

m/z	55.0545	67.0557	71.0481	81.0688	91.055	95.0856	107.0495	119.0849	129.0649	131.0851	145.0998	155.0862	159.0792	169.1022	181.0977	229.1131	241.1893	269.2288	329.2389

Serum

m/z	55.055	67.0544	71.0482	81.0338	91.0549	95.0836	107.0812	119.0841	129.0674	133.0605	145.0651	155.0847	159.0746	169.0999	181.1038	229.193	241.1563	269.1802	329.2424

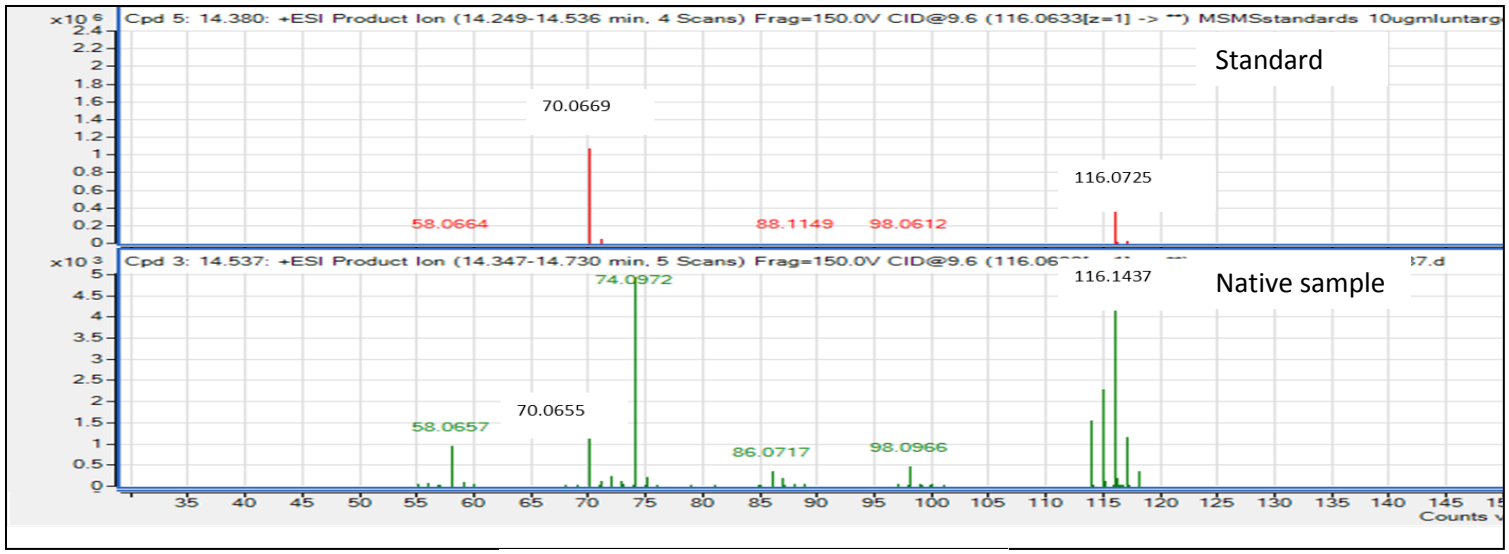
Figure 33. Comparison of spectra of docosahexaenoic acid from a commercially available standard and a native serum. The mass spectrum of the docosahexaenoic acid standard (top) was used to identify the native serum sample (bottom). The compound in the sample was identified by a manual comparison of the m/z values of selected peaks of a commercial standard and a native sample respectively. Some of the peaks are not present in both spectra.



Serum

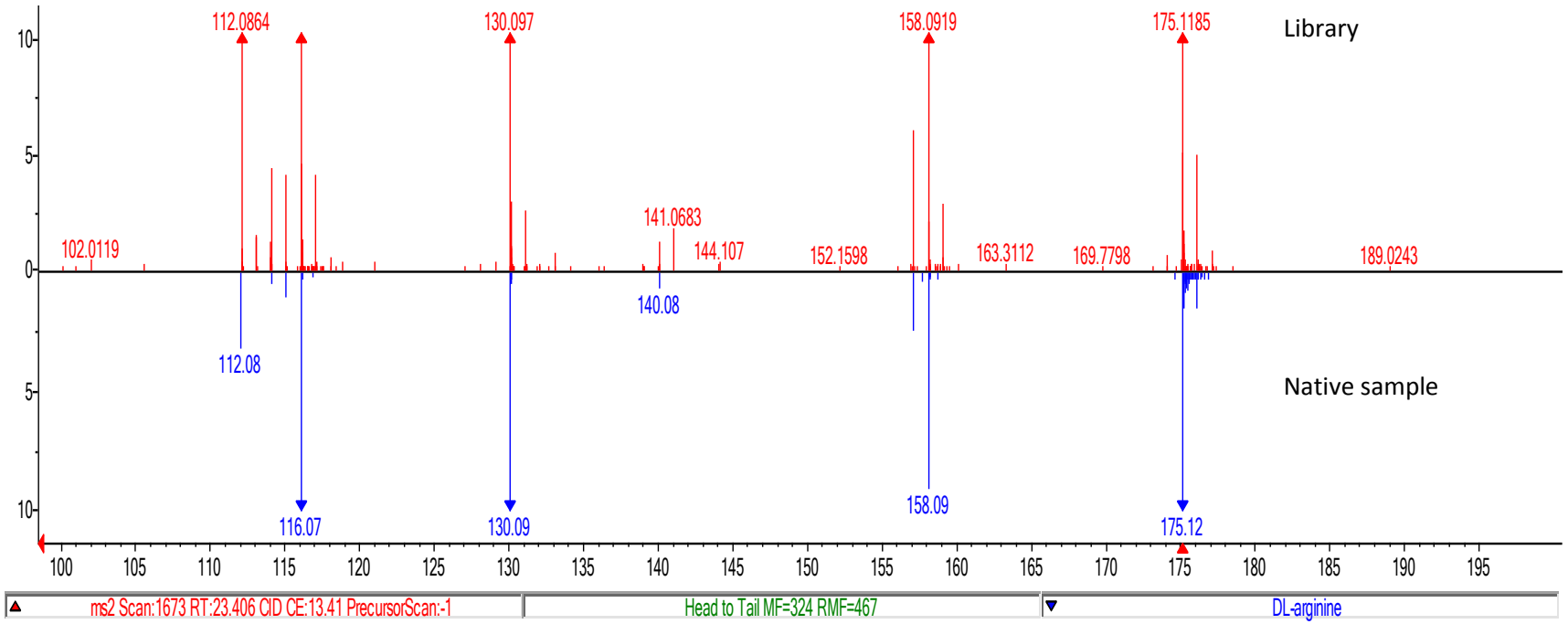
m/z	119.0847	131.0867	151.076	159.1132	173.1288	191.1048	202.0975	225.16	251.0488	260.1511	272.2107	298.2423	311.2249	327.1266	385.3154	400.3383	417.2324

Figure 34. Comparison of spectra of 1,25-dihydroxyvitamin D3 from the METLIN MS/MS library database and a native sample. The mass spectrum of 1,25-hydroxyvitamin D3 acquired from a freely available library (METLIN). The ID score was 73, there were 0 ppm difference between the spectra of the database and the native sample. The spectrum of the native sample was used to identify a candidate metabolite by automatic comparison, spectrum shows the m/z of the SMB identified as the active form of vitamin D3 (1,25 hydroxyvitamin D3), which is the 417 peak, which is preceded in the spectrum by the precursor form of vitamin D represented by peak 400 (25-hydroxyvitamin D3), and also by the precursor isotype of these two, vitamin D3, represented by peak 385. The spectra in METLIN has a Na+ adduct.



Standard				
m/z	116.1409	70.0666	58.0665	98.0606
Serum				
m/z	116.1416	70.0646	58.0645	98.0956

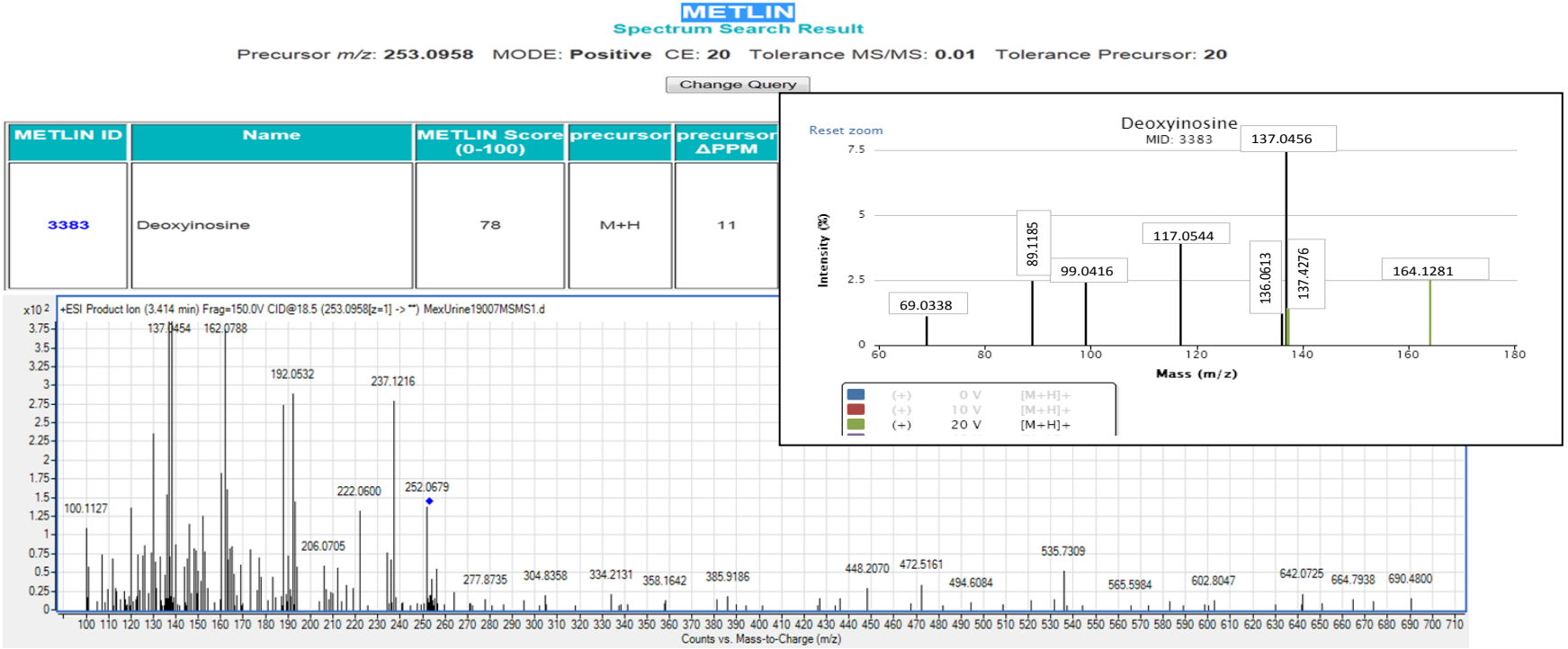
Figure 35. Comparison of spectrum of proline from a commercially available standard and a native serum sample. The mass spectrum of the proline standard (top) was used to identify the native serum sample (bottom). The compound in the sample was identified by a manual comparison of the m/z values of selected peaks of a commercial standard and a native sample respectively. Some of the peaks are not present in both spectra.



10 largest peaks:

116.0703	999.00	175.1185	994.37	130.097	564.87	158.0919	334.49	112.0864	168.88
157.1082	59.47	175.1584	49.96	176.1225	48.65	116.1035	45.67	114.1019	43.26

Figure 36. Comparison of spectra of arginine from the NIST library database and from a urine native sample. The mass spectrum of arginine from NIST spectrum library (red) and the native sample (blue) were compared, this image is zoomed in. The metabolite was automatically identified after the data was imported into the NIST database. The dot-product value for this compound was 621, and the reverse-dot was 771. The peaks with higher abundance that were automatically matched by the library consist of the 116.07 and 175.1 in the library and native sample. Under the figure, the 10 largest peaks matched NIST are presented.



m/z	100.1127	120.0792	130.0488	137.0454	138.0514	162.0788	163.0798	177.0239	188.0557	192.0532	193.0476	222.06	237.1216	252.0679	277.8735	304.8358

Figure 37. Comparison of spectra of deoxyinosine from the METLIN MS/MS library and a native urine sample. Figure represents the mass spectrum of deoxyinosine from the METLIN library (top). This compound was found to be increased in urine of DHF/ND cases compared to ND in Mexican specimens. The metabolite was automatically identified once the data was imported into the METLIN database. The score of the identification was 78 (0-100) and there were 11 ppm difference between compounds from the database and native sample, it should be considered that the ppm difference is relatively high for which the identification of this nucleoside is not unequivocal.

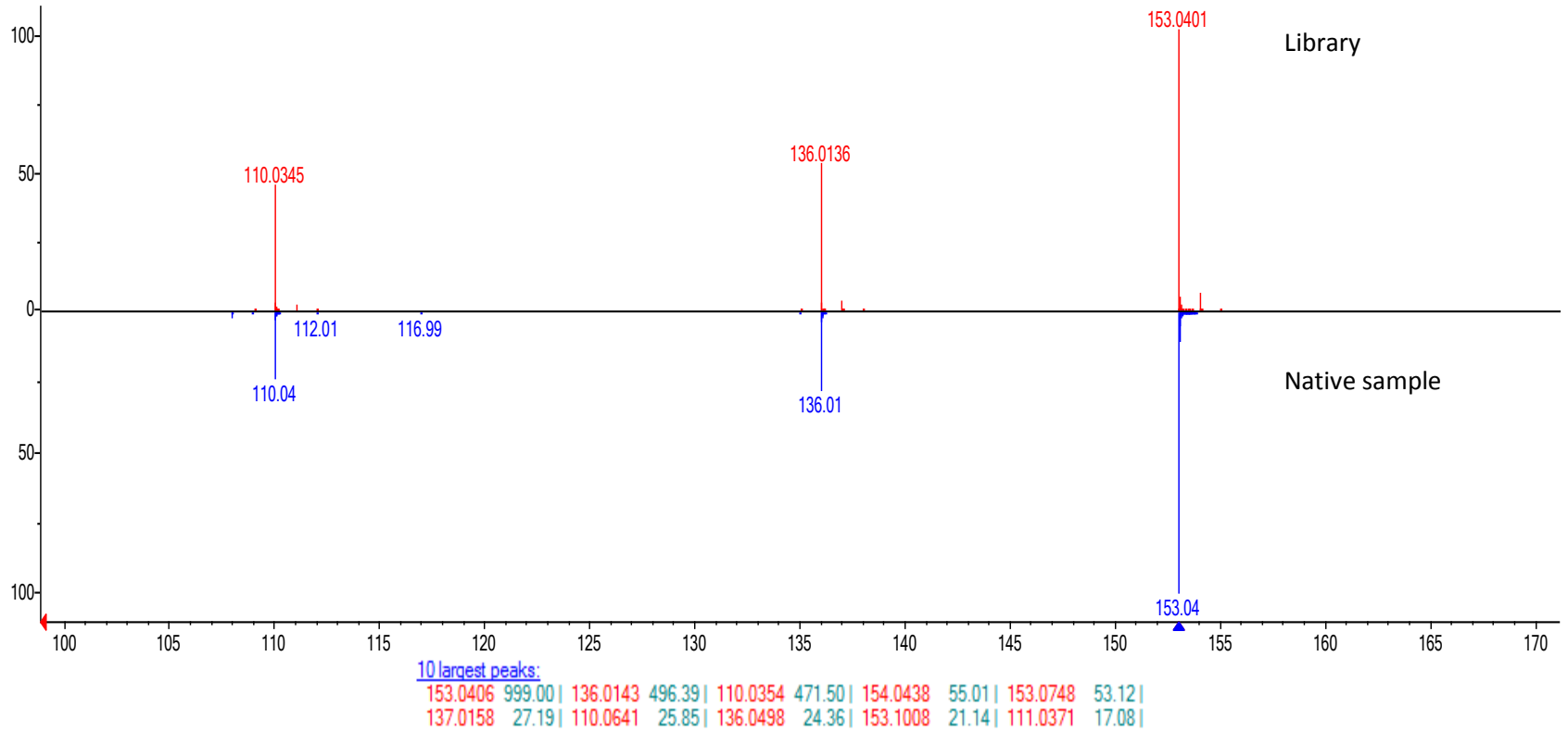


Figure 38. Comparison of spectra of xanthine from the NIST MS/MS database and from a native saliva sample. This figure represents the mass spectra of xanthine from the NIST spectrum library (red) at top and the native sample (blue) at bottom. The metabolite was automatically identified after the data were imported into the NIST database by comparing the 10 largest peaks of the native spectra, which can be seen at the bottom of the figure. The dot-product value for this compound was 767; the reverse-dot was 812. The visible peaks that were automatically matched by the library consist on the *m/z* of 110.0, 136.01, and 153.04.

5.F. DISCUSSION

The main goal of this research project was to identify a panel or biosignature of SMB metabolites that distinguish among the different DEN disease diagnosis groups (DHF/DSS, DF and ND), and that have potential for diagnosis and prognosis of DENV infections using acute phase serum and non-invasive clinical samples.

A portfolio of candidate metabolites that were disturbed in response to DENV infection has been identified by LC-MS/MS. The candidate metabolites identified could differentiate the DEN disease diagnosis groups (DHF/DSS, DF, and ND) by changes in SMB concentration associated with different diagnoses. More studies are needed to determine the role that these metabolites could play in the pathogenesis of infected cells. The putatively identified metabolites might have potential to be exploited for development of point of care (POC) diagnostic tests for DEN diagnosis and prognosis; nonetheless the studies presented in this dissertation only provide data regarding differentiating the disease states in DEN infected patients. Many other compounds identified by LC-MS in serum, urine and saliva (Appendices 1-7) remain to be identified by LC-MS/MS, and some of these will likely be identified as candidate metabolites.

Serum metabolite identifications are overall more reliable than the identifications of non-invasive samples because serum showed less variability than urine and saliva. Data from LC-MS analyses (Chapter 4) evidenced limitations due to sample variability in non-invasive samples, and these limitations need to be considered when assigning identities and interpreting the candidate SMBs in urine and saliva. For example, no SMB or metabolite was statistically significant in both countries in saliva specimens. Molecular features listed in Appendices 6 and 7 could further be analyzed to detect compounds in common among countries. Also, the ppm difference in some identified compounds is relatively large (up to 11 ppm for deoxyinosine), and the spectrum was compared to a library and not to a standard analyzed under the same conditions. Also, the comparison of LC-MS/MS spectra of

commercially available standards with native urine and saliva samples (Table 30 and 31) did not result in identification of any metabolites. The candidate metabolites of urine and saliva presented in Table 34 were identified by comparison of spectra of the native specimens with those provided in libraries; therefore metabolites reported were at level 2 of MSI standard of identification.

In the case of 1, 25 dihydroxyvitamin D3, the identification was made using the METLIN database. Although the score (73) and difference of ppm (0) were acceptable, the visual comparison of spectra from the website and from the native sample was not ideal, the unequivocal identification of this compound should be validated using a commercial standard.

The candidate SMBs identified previously will need to be evaluated for diagnostic efficacy in prospective clinical studies, which are beyond the scope of this study. One (or more likely a number of SMB metabolites) will, when combined with other laboratory and clinical information, hopefully provide a biosignature for DENV infection and for prognosis of progression to severe DEN disease. The identification of the metabolites also will be useful in providing further insights into fundamental disease mechanisms and DENV pathogenesis in human hosts that condition severe disease outcomes. Provocatively, a number of the identified metabolites are associated with metabolic pathways already documented by others to be perturbed during DENV infection of cultured cells, humans, and mosquitoes.[7-9] Clearly many of the metabolites identified are biologically relevant and perturbation of these pathways may be critical in DENV pathogenesis. A summary of the potential biological significance of a number of the candidate metabolites identified in serum, urine and saliva and their potential role in DENV infections follows in the order provided in Table 34.

5.F.1. Alteration in phospholipid metabolism. Two of the candidate metabolites are lysophosphatidylcholines: 1-oleoyl-lysophsphatidylcholine and 1-palmitoyl-lysophosphatidylcholine, (LysoPC(18:1(9Z)/0:0) and LysoPC(16:0/0:0) respectively). These metabolites were increased in acute phase serum of DHF/DSS and DF or ND patients in Mexican and Nicaraguan samples respectively (Table

34). These metabolites are catabolic products of phospholipids. Phospholipids are also precursors of lipid mediators such as platelet activating factors (PAFs) and eicosanoids, which favor inflammation.[294, 295] Several phospholipids were found altered in the pairwise comparisons (Table 24).

Lysophosphatidylcholines (LysoPC or LPC) are products of the hydrolysis of phosphatidylcholine (PC) by phospholipase A1 or A2 (PLA_2) enzymes; lysoPCs only have one fatty acid and therefore are missing an acyl chain. PLA_2 cleaves the phosphatidylcholine to yield a fatty acid and lysophosphatidylcholine, which is involved in alteration of membrane structures and mediation of inflammation. [296-298] The fatty acid components of the identified lysoPCs were oleic acid (C18:1) and palmitic acid (C16:0).

Phospholipids and fatty acids (such as α-linolenic (C18:3) acid and docosahexaenoic acid (C22:6)) are normally found in the lipid bilayer of cellular membranes. An increase in the concentration of released lysoPCs has been postulated to enhance transmembrane permeability by the presence of surfactants in the vicinity of the membrane. LysoPCs can act as the surfactants that lower passive transmembrane barriers in biological membranes. Integration of lysoPCs or fatty acids into the lipid membrane can facilitate the formation of small-scale phase separated structures, which can increase transmembrane permeability, this is associated with the formation of “leaky” boundaries between cells. [299] Indeed, lysoPC conditions vascular endothelium barrier dysfunction, and lysoPC concentrations increase under inflammatory conditions by endogenous GPR4 (G protein-coupled receptor family 4) in endothelial cells, which indicates that GPR4 could play a critical role in the inflammatory responses activated by lysoPC. [289]

DENV replication is intimately associated with host cell lipid biosynthesis and metabolism. DENV replication complexes are enclosed in intracellular membrane vesicles that facilitate the exchange of components with the cytosol for genome replication and virus assembly and shield PAMPS such as

dsRNA from detection.[8, 299] Palmitic acid has been shown to facilitate the incorporation of lysoPCs into DENV-infected mosquito cells; palmitic acid and stearic acid are normally found to be increased in DENV-infected cells.[8] This is consistent with the results obtained in this study; the concentrations of this fatty acid coupled to lysoPC (1-palmitoyl-lysophosphatidylcholine) were found to be increased in DEN disease.[300]

DENV is known to rearrange cellular membranes to facilitate viral genome replication. It has been shown that DENV-induced vesicles, convoluted membranes, and virus particles to be endoplasmic reticulum-derived. Invaginations of the ER membrane are induced likely by NS4A, along with other viral and cellular factors and give rise to membranous vesicles that are connected to the cytosol via a pore.

Analysis revealed an increase in fatty acids such as palmitic acid (C16:0) and oleic acid (C18:1) coupled to lysoPCs, which suggests that several metabolic pathways may be significantly altered during DENV infection as DENV stimulates *de novo* phospholipid biosynthesis to enclose the replication complex in membranes(Figure 39). [8, 9] Phospholipid biosynthesis can be increased in DENV- infected cells; the prevalent phospholipids found to be increased primarily contain C16 and C18 unsaturated acyl chains.[7, 8] This would be consistent with our results in which two lysoPCs carrying those fatty acids were significantly increased in serum of DEN patients (Table 34).

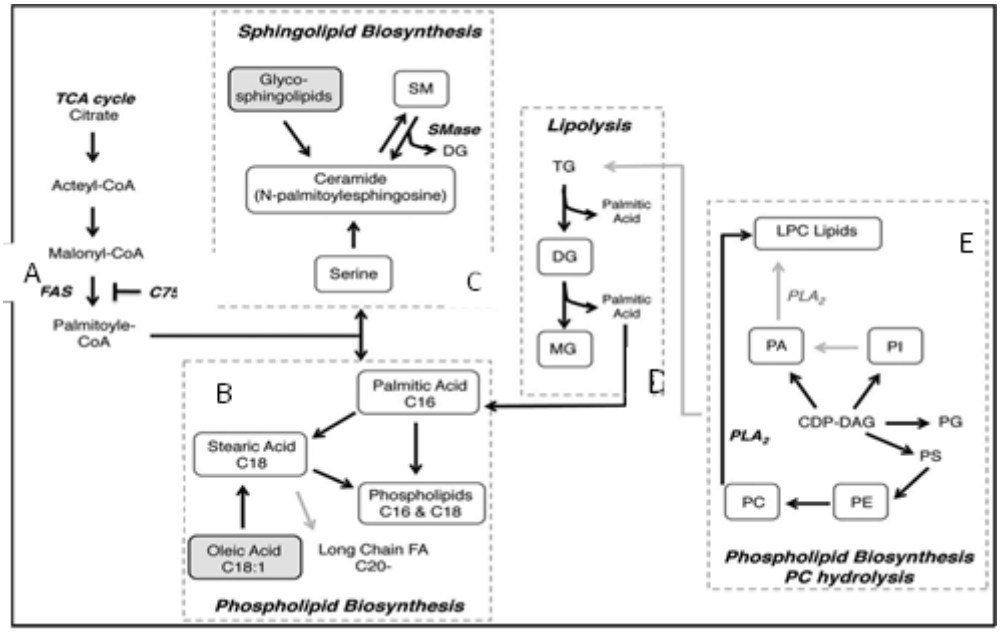


Figure 39. Altered phospholipid biosynthesis. A.DENV infection promotes the biosynthesis of phospholipids. B. Increase of lysoPCs containing oleic acid and palmitic acid was observed in the results. Interestingly, in DENV infected mosquito cells the prevalent phospholipids increased are the same as the ones found in this study (Table 34). C. De novo sphingolipid biosynthesis is influenced by the increased lipid biosynthesis caused by DENV. D. Lipolysis has been postulated to be increased in DENV infected cells, which generates palmitic acid. E. increased levels of lysoPC in DENV infected cells suggests activation of PC hydrolysis by PLA2 . Abbreviations: SM, sphingomyelin; CER, ceramide; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; lysoPC, lysophosphatidylcholine; PLA2, phospholipase A2; PA, phosphatidic acid; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylcholine. Taken from Perera, et al.[8]

The pathways altered by DENV in infected mosquito cells are pictured in Figure 39; oleic acid and palmitic acid concentrations are shown increased by DENV infection, as well as lysoPC production. This is relevant to the results presented because lysoPC also has the ability to affect endothelial cells in diverse manners (endothelial cell dysfunction is postulated as one of the causes of plasma leakage in DHF/DSS). LysoPC has been shown to induce the production of growth factors, adhesion molecules and MCP-1 and IL-8 (chemotactic factors for monocytes, neutrophils and lymphocytes) in microvascular endothelial cells and in large vessels.[298, 301] RANTES (regulated on activation, normal T cell expressed and secreted) is known to be induced in microvascular endothelial cells by lysoPC.[298] This suggests that lysoPC can be a mediator of acute and chronic inflammation and regulate pathophysiological events

throughout the vasculature and locally at tissue sites. LysoPC can also accelerate pathogenic inflammation and increase intracellular reactive oxygen species in endothelial cells that can damage these cells.[288, 298, 302]

LysoPC is a pro-inflammatory metabolite that participates in pathophysiological events in vascular endothelium; thus increased concentrations of lysoPC in serum could play a critical role in the pathophysiology of vascular leakage which is the key characteristic of DSS. In summary, demonstration that lysoPCs were metabolites that differentiated the DEN diagnostic groups and thus likely played a role in DENV pathogenesis was consistent with previous reports that demonstrated perturbation of lysoPCs in DENV infected mosquito cells and in DENV-infected human serum.[7, 8]

5.F.4. Alterations to α-linolenic acid (ALA) metabolism. α-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid) was found to be increased in DHF/DSS vs. DF and DHF/DSS vs. ND in Nicaraguan serum samples.

ALA is an essential polyunsaturated omega-3 fatty acid (PUFA) that contains an 18-carbon chain and three *cis* double bonds. The first double bond is at the third carbon from the omega end of the fatty acid chain; thus this compound is considered an omega-3 fatty acid. ALA is a precursor of docosahexaenoic acid (Figure 40), which was found to be increased in this analysis. The principal source of ALA is dietary. Omega-3 fatty acids are considered to have anti-inflammatory effects on eicosanoids produced from arachidonic acid (AA) Figure 40 shows the origin of ALA and DHA by diet, its transformation into intermediary products and finally their conversion into resolvins and lipoxins. It also shows the prostaglandins of series 2 and 3. Series 2 prostaglandins (PGs) are produced from a 20-carbon unsaturated fatty acid or arachidonic acid. The series 3 PGs are produced for eicosapentaenoic acid. Series 2 PGs are involved in inflammation, clotting, dilation and swelling. Series 3 PG have a modulator effect and can act as anti-inflammatory compounds. [303, 304]

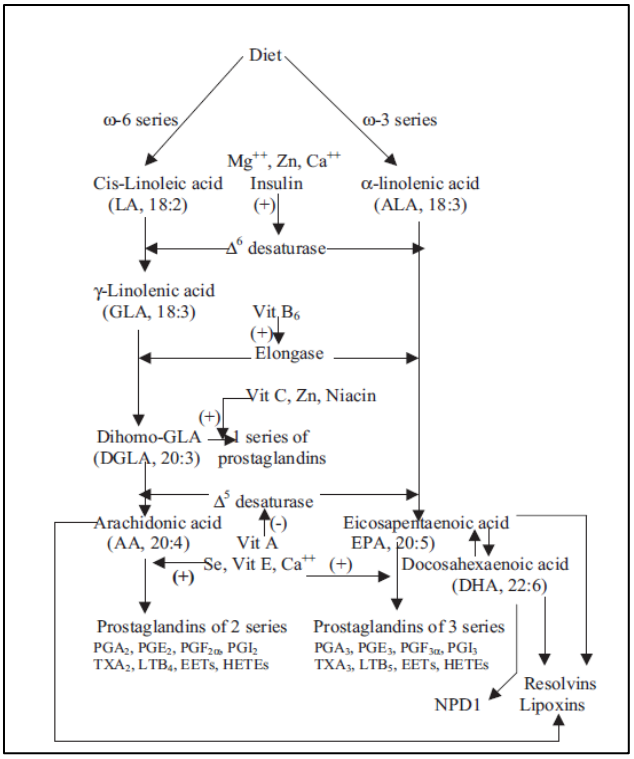


Figure 40. Origin of ALA and metabolic pathway linking it to docosahexaenoic acid and prostaglandin biosynthesis. Figure illustrates the metabolism of ALA and docosahexaenoic acid and the formation of prostaglandins. Taken from Das, 2006.[304]

PUFAs are critical components of membranes, and play important anti-inflammatory and inflammatory resolution roles; they also have important roles in cell integrity, development, maintenance, and function. ALA is particularly important in regulating membrane function; cell-membrane fluidity is determined by its lipid composition. ALA confers on membranes properties of fluidity, and determines and influences the membrane-bound enzymes and receptors.[304, 305]

In hepatocytes, ALA is transported to the endoplasmic reticulum, where it is transformed into eicosapentaenoic acid (EPA) and tetracosahexaenoic acid that are then directed to peroxisomes (Figure 41), where converted in docosahexaenoic acid (DHA) which can be released.[305, 306] DHA was also found to be increased (Table 34). DENV infection can induce liver cell damage.[307] DENV infection could alter hepatocyte metabolism and with this, potentially be a contributing factor for elevated levels of ALA and DHA in serum.

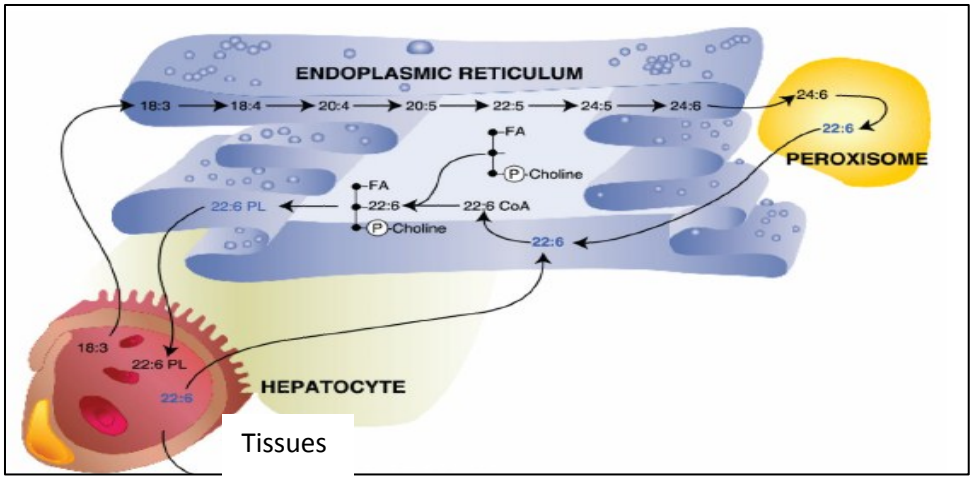


Figure 41. ALA and DHA metabolism within the hepatocyte. ALA (18:3) and DHA (22:6) are transferred to the endoplasmic reticulum where DHA is attached to a n-2 position of phosphatidylcholine to form a phospholipid prior to release to the circulatory system. Taken from Bazan et al.[305]

Dietary intake of this compound can lead to decreased platelet aggregation, although the mechanism is not clear. [306, 308] ALA can inhibit TNF-α-induced endothelial tissue factor expression at the transcriptional level,[306] and can suppress IL-1, IL-2, and IL-6 (proinflammatory cytokines) and thus functions as an endogenous anti-inflammatory molecule [304] these effects potentially could have a protective role during DEN disease against progressing to DSS.

ALA was found in this analysis to be increased in serum of patients with DHF/DSS. The elevation of this metabolite in serum could be due to damage to the DENV-infected cells, (potentially hepatocytes), thereby releasing this compound, which would cause an elevation in serum levels of ALA. There are no current studies on DENV infections and ALA metabolism. Clearly, further studies are needed to corroborate this hypothesis.

5.F.5. Perturbation of docosahexaenoic acid (DHA) metabolism. DHA (4Z,7Z,10Z,13Z,16Z,19Z-docosahexaenoic acid) was found in increased concentrations when comparing DHF/DSS patients to DF patients and DHF/DSS patients to ND patients in Nicaraguan serum samples. (Table 34) It is an essential PUFA derived from ALA and is also classified as Omega-3 (Figure 40). It is the most abundant of the long-chain polyunsaturated omega-3 PUFAs, and it has important roles in the central nervous system

(CNS) and the cardiovascular system. DHA is activated and esterified by phospholipase A_2 (PLA_2) into sn-2 phospholipids, mainly phosphatidylcholine. (Figure 41 and 42) [305] Docosahexaenoyl-phospholipids found in cellular membranes are critical due to the biophysical implications of these molecules for membrane protein organization and specifically for their roles in neurotransmission and endocytosis.[305]

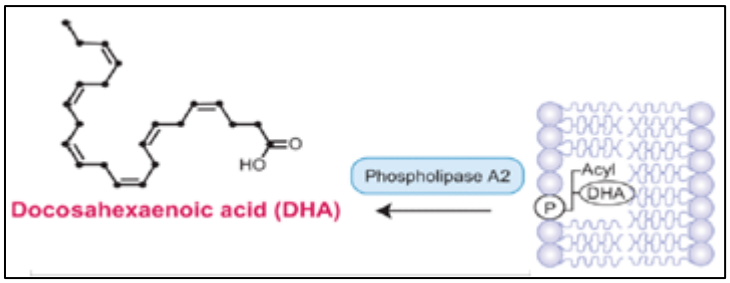


Figure 42. DHA can be found in phospholipids in cell membranes. Membrane phospholipids containing a docohexaenoyl chain at sn-2 are hydrolyzed by phospholipase A2, generating free DHA. Taken from Bazan et al. [305]

DHA, via the action of lipoxygenases it is the precursor of the docosanoids, termed 'resolvins' or 'protectins', which are analogous to the eicosanoids but have potent anti-inflammatory and immuno-regulatory actions. DHA is believed to have specific effects on gene transcription that regulate a number of proteins involved in fatty acid synthesis and desaturation.[304, 305] DHA has anti-inflammatory properties in contrast to proinflammatory properties of other omega-6 PUFAs. This compound is a precursor of several key mediators of inflammation, including PGE3, a cyclooxygenase (COX) metabolite of AA. PGE3 are believed to be less inflammatory than other PGs and less efficient inducing IL-6. [303]

As with other PUFAs (such as ALA), DHA also has important roles in cell integrity, development, maintenance, and function.[305] DHA has been postulated to inhibit oxidative stress induced in endothelial cells by calcium influx by alteration of lipid composition. During endothelial dysfunction, calcium overload of the plasma membrane is one of the principal pathophysiological changes that

damages endothelial cells irreversibly. DHA has the ability to inhibit the oxidative stress induced by calcium influx by changing the raft lipid composition.[309]

Consistent with the results presented, DHA was found to be increased in cases with severe outcomes (DHF/DSS). DENV-induced damage to endothelial cell membranes could have caused the release of DHA from the phospholipids normally maintaining the integrity of the membranes of these cells, causing DHA to be detected in high abundances in serum.

5.F.2. Perturbations in Vitamin D3 metabolism. Vitamin D3 isotypes were detected in altered concentrations in serum specimens in every analysis performed. Different isotypes statistically differentiated the diagnosis groups. 1, 25-dihydroxyvitamin D3 was a candidate metabolite identified by comparing the mass spectrum from a native sample and an online database (METLIN). 1,25 dihidroxyvitamin D3 was identified when comparing DHF/DSS patients to DF patients in Nicaraguan samples and in DHF/DSS patients when comparing them to ND patients and DF patients when comparing them to ND patients in Mexican samples. This metabolite exhibited a negative FC or a decreased concentration in severe DEN cases (Table 34).

Active forms of vitamin D have been shown to be obtained by diet or UV-mediated synthesis in the epidermal layer of the skin, where 7-dehydrocholesterol is converted to pre-vitamin D3 secondary to UV exposure. The pre-vitamin D3 from the skin or from dietary sources binds to vitamin D-binding protein in the circulation and is transported to the liver, where it is hydroxylated. From this process, 25, hydroxyvitamin D3 is created and then further hydroxylated in the kidney by 1α- hydroxylase, which generates the active form 1, 25-dihydroxyvitamin D3.[106] There is now evidence that human endothelium is able to produce active vitamin D by the action of 1α-hydroxylase activity induced by inflammatory cytokines. Thus the active form, 1, 25 dihydroxyvitamin D3 could be a modulator of endothelial cell function and adhesion by a paracrine/autocrine function in the organs in which is synthesized. *In vitro* studies have demonstrated that the synthesis of 1, 25- dihydroxyvitamin D3 by

endothelial cells is stimulated by inflammatory cytokines and it could be a modulator of leukocyte adhesion. [310]

1, 25-dihydroxivitamin D3 is the active form of vitamin D which has immunoregulatory capabilities over immune cells, including monocytes, macrophages, dendritic cells, and T and B lymphocytes of the innate and adaptive immune responses. These immune cells can express vitamin D-activating enzymes, which allow inactive vitamin D to be converted into the active form.[106, 310] Vitamin D receptors (VDRs) can be found in most immune cells; provocatively CD4+ and CD8+ T cells, B cells, neutrophils, and antigen-presenting cells (APCs) such as macrophages and dendritic cells.[311] 1, 25-dihydroxyvitamin D3 also functions in regulating T helper cells (Th1) and in inducing regulatory T-cell function.[312] 1, 25-dihydroxyvitamin D3 modulates T cell and macrophage function through a nuclear receptor that acts as a transcription factor by binding to vitamin D3 and affecting other transcription factors. [313] 1, 25-dihydroxyvitamin D3 stimulates the innate immune response by enhancing the chemotactic and phagocytic responses of macrophages. In APCs, this vitamin inhibits the expression of MHC-II and its co-stimulatory molecules, also the production of IL-12 and IL-23, with this shifting the polarization of T cells from a Th1 and Th17 phenotype towards a Th2 phenotype acting as potential anti-inflammatory compound (Figure 43). [106]

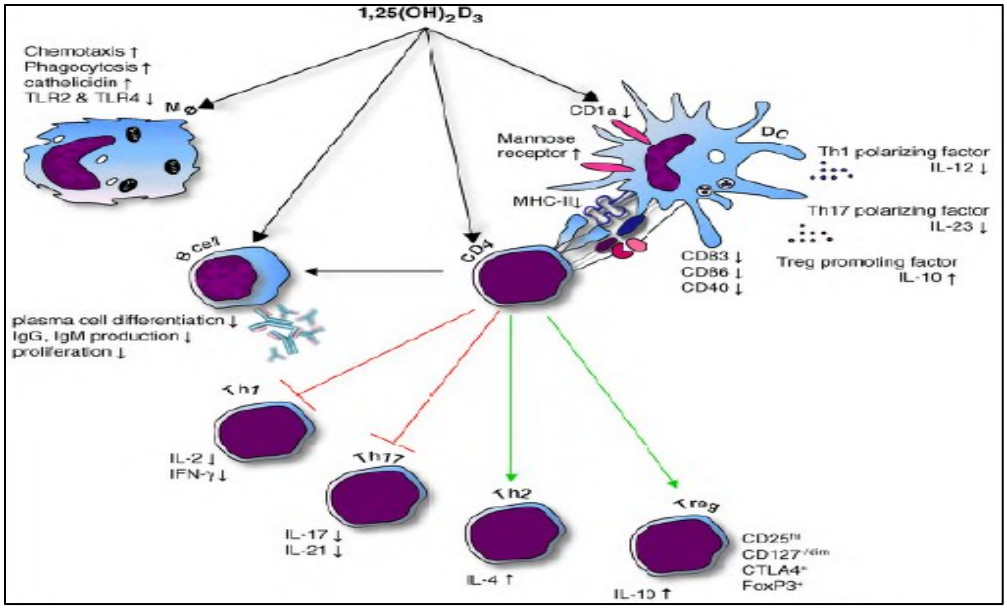


Figure 43. Immunomodulatory effects of 1,25-dihidroxyvitamin D3. 1, 25-dihidroxyvitaminD3 stimulates innate and adaptive immune responses. It increases chemotactic and phagocytic responses of macrophages and antimicrobial proteins and it also polarizes T cells towards a Th2 responses and inhibiting Th1. This compound also blocks plasma-cell differentiation IgG, IgM production and B-cell proliferation. Taken from Baeke et al.[106]

Augmented synthesis of 1, 25-dihydroxyvitamin D3 by endothelial cells could have potentially a modulatory effect on production of inflammatory cytokines in vascular endothelial cells.[310] The 1, 25-dihydroxyvitamin D3 was significantly decreased in DEN disease. In the specimens diagnosed with severe forms of disease (DHF/DSS), disruption of homeostasis in the endothelial cells and therefore in the metabolism of this compound could result in the lower concentrations found in serum specimens of DENV infected patients.

1, 25-dihydroxyvitamin D3 was decreased in DENV infected Nicaraguan and Mexican patients; in the Nicaraguan patients, it was decreased in the severe disease group (DHF/DSS), but in Mexican patients there was no distinction by disease severity (Chapter 4, Tables 21 and 22). It is worth mentioning that the Nicaraguan patients were all children, who are more likely to develop DHF/DSS. Perhaps this is the reason that this metabolite was identified in the pairwise comparisons of the results of the analyses of specimens from the Nicaraguan patients.

1, 25-dihydroxyvitamin D3 could be considered as one of the most biologically significant candidate metabolite detected in this metabolomic project due to the differentiation of disease states and its statistical significance in both countries. Its incorporation into a POC test could be difficult due to the decrease of this metabolite in DHF/DSS patients (Table 34). Interactions of this metabolite with endothelial cells and the decreased observed in immune diseases by others[106, 312] prompts speculation about the potential role of decreased concentrations in patients presenting DHF/DSS. Further studies are needed to determine the role that this compound plays in the metabolism of DENV-infected cells.

5.F.3. Perturbation of proline metabolism. Proline exhibited a negative FC or decreased abundance in the three pairwise comparisons in Nicaraguan serum samples (Table 34). Proline is an α-amino acid; it is not an essential amino acid, which means it can be synthesized by humans. Proline can act as an immune-modulator of the intracellular redox environment, protecting mammalian cells against oxidative stress, and thus it can be considered an antioxidant.[314]

Proline is oxidized to glutamate by mitochondrial enzymes proline dehydrogenase (PRODH) and Δ1-pyrroline-5-carboxylate (P5C). PRODH is activated by p53 (regulates cell cycle) with proline oxidation (Figure 44), which causes an increase in intracellular reactive oxygen species (ROS) and induction of cellular apoptosis *in vitro*. Overall, proline has a role protecting the cells against oxidative-stress. [315, 316]

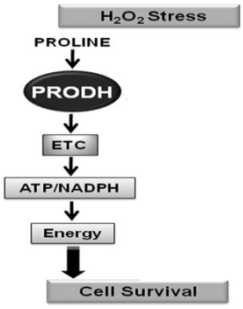


Figure 44. Proline protection against oxidative-stress. Proline oxidation is coupled to reduction of the electron transport chain (ETC) by PRODH, this enzyme supports oxidative phosphorylation and ATP

formation and prevents decreases in NADPH/NADP, which sustains cells during oxidative stress. Taken from Natarajan et al.[316]

Metabolomics analyses have shown that intracellular amino acids are altered in endothelial cells in response to oxidative-stress. Proline was found to be elevated due to metabolic stress in hyperglycemic endothelial cells, suggesting a role of this amino acid in the oxidative stress pathway. As a potential antioxidant, the concentration of proline was increased potentially to protect cells from further damage.[283] Proline is known to influx into endothelial cells during osmotic stress and to form part of a cellular transport system that can be affected in hypertonic stress.[317] Patients with DENV infections are frequently dehydrated due to fever, vomiting, and anorexia, which could lead to hypertonic stress.

In this regard, proline was demonstrated to be decreased in serum of DEN patients (Table 34). Previous research [283, 285, 317] has demonstrated that proline is altered in endothelial cells. Unfortunately there are no previous metabolic studies on proline metabolism done in DENV-infected cells or DEN patients. The hypothesized molecular mechanisms underlying the role of proline in DENV infected serum still needs to be investigated.

5.F.5. Alterations in arginine metabolism. Arginine was increased in DHF/DSS patients when compared to DF patients and in DHF/DSS patients when compared to ND in Mexican urine specimens (Table 34). It is an α-amino acid and is one of the most common natural amino acids. It is a semi-essential amino acid, and the human body will produce enough under healthy conditions.

The transporter system for arginine is co-located with nitric oxide synthase (eNOS), which suggests that NO production could be regulated by arginine.[318] (Figure 45)

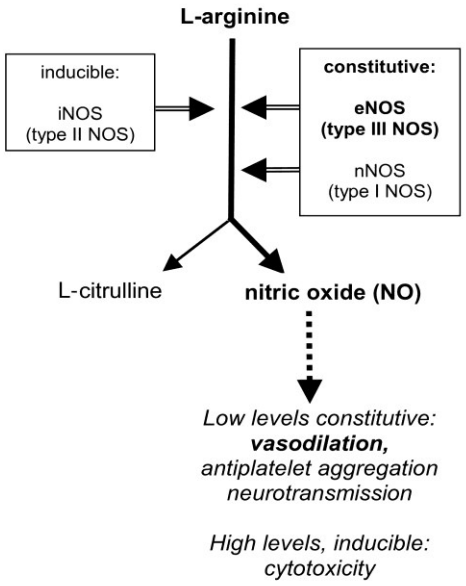


Figure 45. Arginine-nitric oxide pathway indicating the effects of NO in tissues.

Disease-induced alterations in serum-levels of arginine and other amino acids modulate vascular relaxation, based on the fact that arginine is the physiological precursor for nitric oxide biosynthesis.[319] The arginine-nitric oxide signaling pathway could be one of the key second messengers systems that regulate vascular tone and permeability.[318, 320] Arginine can also inhibit platelet aggregation.[321]

Nitric oxide (NO) has physiological and pathological functions and can modulate homeostasis in many different types of cells. It is a modulator of platelet function and has the ability to inhibit aggregation, recruitment and adhesion to the vascular endothelium.[319] NO is synthesized by the oxidation of arginine and catalyzed by NO synthase (NOS) enzymes. Platelets express the inducible and constitutive isoforms of NOS, and importantly, arginine transport is the rate-limiting step in NO production. Arginine is transported predominantly by platelets, and the system that transports arginine in the platelets also mediates the Na^+ independent cationic and Na+ dependent neutral amino acid transport. [318]

Mendes-Ribeiro et al [322] have postulated that arginine could play a role in DENV pathogenesis. In the early stages of DENV infection, a systemic host inflammatory activation response occurs. NO has a key role in the inflammatory processes and during acute infection, it is increased. Increased NO activity (anti-aggregatory for platelets) could condition hemorrhagic and vascular leakage manifestations in severe DEN disease. These anti-aggregatory manifestations, which are likely triggered to the immune response by DENV infection, could be attributed to the enhancement of the arginine-NO pathway. [322]

The kidneys have a critical role in arginine metabolism by synthesizing it *de novo* and by eliminating the methylated arginine by urinary excretion. In the results shown, arginine's urinary excretion was increased in hemorrhagic cases (Table 34). This could be due to an excess of arginine in plasma that was cleared by urinary excretion or potentially to kidney compromise due to DENV infection. Therefore, the increase of arginine excretion in urine of patients with DHFSS could be due to increased arginine stimulated by the immune system activation of NO. This would have consequences on platelet aggregation that could worsen DHF manifestations.

5.F.6. Perturbation of deoxyinosine metabolism. Deoxyinosine was elevated in DHF/DSS vs. ND in Mexican urine specimens (Table 34). It is a nucleoside product of hypoxanthine generated by the action of adenosine deaminase. It is a nitric oxide-derived DNA adduct. [323] Deoxyinosine is normally found in urine and is commonly used as a biomarker for DNA damage.[324] In chronic inflammation there is a constant generation of macrophages; deoxyinosine is primarily produced by nitric oxide. Therefore an increase of NO during the immune response in DEN disease could result in elevation in the levels of deoxyinosine found in urine. There is no previous research on the role of this candidate metabolite in infectious diseases. Further analyses are needed to determine the role of this metabolite in DEN disease and pathogenesis.

5.F.8. Perturbation of xanthine metabolism. Xanthine was found elevated in DHF/DSS patients when compared to ND patients and DF patients when compared to ND patients in Mexican saliva specimens (Table 34).It is a purine base found in most body tissues and fluids. It has been reported that an increase in xanthine levels could be due to cell damage in a radiation-exposure study in rats. [325] This compound has been detected in serum of newborns and quantified in order to predict inborn errors of metabolism,[326] and also has been found to be elevated in serum after rigorous exercise.[327] Metabolic stress leads to acceleration of adenine nucleotide degradation, which produces hypoxanthine and xanthine dehydrogenase activity. The hypoxanthine is converted to xanthine and uric acid by the action of xanthine dehydrogenase, which leads to an increase of xanthine in plasma. [327] There are no reports, to the best of my knowledge, on the effects of xanthine on infectious diseases, endothelial cells, or DENV infection. However several studies have investigated the influence of the precursor of this metabolite or the enzyme that degrades it on disease. The study by Kaya, et al,[327] suggested that elevated xanthine in saliva samples could be due to metabolic stress in the organism. Further studies are needed to understand the role of this compound in DENV pathogenesis.

5.F.9. Summary. A total of nine candidate metabolites whose concentrations were changed significantly in DEN disease patients have so far been identified with LC-MS/MS analysis: Six in serum, two in urine, and one in saliva. These candidate metabolites included phospholipids, fatty acids, nucleosides, vitamin D isotypes and amino acids. These candidate metabolites are indicative that there are measurable metabolite differences among DEN diagnosis groups that can be detected by metabolomics studies.

There are likely many more candidate SMBs that will be identified in serum as well as in non-invasive samples (urine and saliva) by comparison by LC-MS/MS of spectra of native samples and purchased standards or with spectra from NIST and METLIN databases. Indeed hundreds of molecular features remain to be analyzed by LC-MS/MS in each of the types of samples (Appendices 1-7).

The candidate metabolites were investigated to determine their potential biological and physiological roles in in DENV pathogenesis. Some of the candidate metabolites are provocative in terms of DENV replication and pathogenesis. For example, some of the candidate metabolites are involved in cell homeostasis, whose disruption could be directly involved in DENV infection and replication (e.g. fatty acids). Others (e.g., lysoPC) have been shown to be involved in endothelial cell barrier function, which is provocative in the context of vascular leakage and shock in DSS. Others (e.g., vitamin D3) are associated with endothelial cell function and immune response, which could also be altered during DENV replication. These potential mechanisms and pathways need to be investigated in more detail in order to confirm their potential roles in DENV pathogenesis. Targeted *In vitro* studies of metabolites identified could be informative in this regard. Because DENV must infect and replicate in mosquitoes, comparative targeted metabolomics studies in the vector or in vector cells could also be informative about fundamental mechanisms that condition DENV replication and disease production. Indeed, metabolomics studies of DENV infection in mosquito cells identified many metabolites that were differentially expressed in infected versus non-infected mosquito cells and many of the metabolites were similar to those that were perturbed DENV infected vertebrate cells and in humans.[8]

Newer strategies for candidate metabolite identification are based on the comparison of MS/MS spectrum of the targeted compound with those in online libraries and databases. Laboratories are now developing their own libraries in addition to commercially available ones for metabolite identification. This method is less expensive for compound identification because there is no need to purchase and analyze the commercial standard. Compounds are identified by matching of spectrum of native sera with the spectrum available in a library.[328, 329] Nonetheless, the Metabolomics Standard Initiative (MSI) considers this type of identification of level 2 accuracy; in contrast, standard-based identification is considered level 1. Further studies need to be performed using mass spectrum libraries to validate this identification strategy.

In summary, LC-MS/MS identified a panel of candidate metabolites that differentiate DEN disease outcomes (i.e., DHF/DSS, DF, ND) and a search of the literature has revealed that many of these are potentially biologically and physiologically relevant in DENV infection and pathogenesis in humans. This panel of candidate SMBs, as well as many more candidate metabolites that will be identified in ongoing LC-MS/MS studies will be investigated for diagnostic and prognostic utility using acute phase specimens in metabolomics-based clinical studies in Merida and Managua.

The candidate metabolites will also be evaluated in terms of potential development of clinically relevant POC assays that could be readily applied in clinics where most of the patients present. The ultimate goal is to develop a POC diagnostic test to detect selected candidate metabolite or metabolic pathway perturbations that predict progression to severe DEN disease and to adapt this test for assay of serum as well as non-invasive clinical samples. Results from such a POC could be incorporated into diagnostic algorithms including other laboratory and clinical biomarkers for improved diagnosis and prognosis of DENV infections. Critical prognostic information will be forthcoming that will allow focus of clinical care and intervention on those at greatest risk for severe DEN disease. Obviously these translational research studies are beyond the scope of this dissertation. Nonetheless, my studies have revealed the potential for application of metabolomics to understanding the fundamental pathogenic mechanisms that condition DENV infection and disease production in humans and have provided a panel of candidate metabolites that differentiate the DEN disease diagnostic groups (DHF/DSS, DF, and ND).

CHAPTER 6

SUMMARY, FUTURE DIRECTIONS, AND CONCLUSIONS

6.A. SUMMARY OF RESULTS

Dengue (DEN) is the most important viral disease of humans transmitted by mosquitoes. Dengue virus (DENV) is transmitted to humans principally by *Ae. aegypti*. Epidemics of dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) have escalated in frequency, and DEN is one of the leading causes of morbidity and mortality in endemic countries. The needs and opportunities to address this globally important disease are many.

This dissertation research has provided novel approaches and tools for addressing the DEN pandemic, both by adapting a commercially available DENV NS1 antigen capture test to provide improved mosquito-based surveillance for impending DENV epidemics and by providing an exciting new metabolomics approach with great potential for DENV diagnosis and prognosis of patient progression to severe disease using acute phase serum, saliva, and urine. Limited studies were also conducted to determine the potential for DENV NS1 antigen detection in acute phase urine and saliva samples for DEN diagnosis. The results of these studies and pertinent lessons learned (where appropriate) will be reviewed briefly, and future directions in each of the areas will be proposed.

6.A. DETECTION OF DENV NS1 ANTIGEN IN INFECTED *Aedes aegypti* USING A COMMERCIALLY AVAILABLE KIT.

6.A.1. Brief summary of results. In the absence of vaccines, DENV surveillance and control programs are the best approach to prevent DENV infections. Passive surveillance of DEN cases in humans has proven to be too slow to effectively intervene in impending epidemics. Mosquito-based surveillance could be useful in this regard. The studies and results provided in Chapter 2 strongly suggest that NS1 is an exceptional analyte for detection of DENV in mosquitoes. The commercially available NS1 antigen detection test developed to test serum (Platelia™ Dengue NS1 Ag) proved to be an easily used, rapid,

sensitive and specific test for detection of DENV in mosquitoes infected by intrathoracic injection with DENV-2. NS1 antigen was detected in 98% of infected mosquitoes. The test readily detected one infected mosquito in pools of up to 1000 mosquitoes (Table 1). The test also detected one infected mosquito in pools of mosquitoes subjected to desiccation cycles and freeze-thaw cycles, with the purpose of mimicking conditions likely encountered in mosquito-based field surveillance programs. The NS1 antigen test was more sensitive than RT-PCR and virus isolation for detecting infected mosquitoes (Table 5). The results indicated that the NS1 test constitutes a very promising approach for mosquito based surveillance.

6.A.2. Future directions. Clearly the Platelia™ Dengue NS1 Ag kit, has proven to be effective for DENV-2 antigen detection in *Ae. aegypti*. Further laboratory studies should determine the sensitivity and specificity of the test to detect the other three DENV serotypes in *Ae. aegypti* as well as to detect all of the DENV serotypes in infected *Ae. albopictus* mosquitoes, the second most important vector of DENV.

However, before being integrated into DENV surveillance and vector control programs, *bonafide* field trials in DEN endemic areas need to be conducted to determine the diagnostic efficacy of the test in real world conditions. Such studies should determine the sensitivity and specificity of the test for detection of each of the four DENV serotypes using actual field collected specimens. Such studies will reveal the effect of true field environmental conditions (e.g., ambient temperature and humidity) and contaminants (e.g., fungi and bacteria) on test performance. The NS1 antigen test should be compared with RT-PCR and virus isolation both in terms of sensitivity and specificity as well as cost and time for DENV detection in field caught mosquitoes. These studies will reveal the true diagnostic efficacy of the test and the potential for improving DENV surveillance and control in public health programs.

6.B. NS1 ANTIGEN DETECTION IN NON-INVASIVE SPECIMENS FOR DEN DIAGNOSIS.

6.B.1. Brief summary of results. The potential for detection of DENV NS1 antigen in non-invasive clinical specimens was investigated. A commercially available test was used to detect NS1 antigen in

serum, urine and saliva samples. DENV NS1 was detected in serum as well as in urine and saliva, but at a significantly lower rate in the non-invasive samples (Table 7). The specificity of the test was 100% in serum and urine. Interestingly, NS1 was detected more frequently in saliva than in urine samples from infected individuals (Table 7). However, the test as currently constructed is not recommended for diagnosis of DENV infections in non-invasive samples.

6.B.2. Future directions. Although NS1 was detected in urine and saliva of DEN diseased patients, the sensitivity was low. Nonetheless, the results were encouraging, because potentially the Platelia™ Dengue NS1 Ag kit could be optimized for NS1 antigen detection in urine or saliva, or the sample preparation could be improved for non-invasive clinical specimens. For example, drying the sample (saliva or urine) and reconstituting it at a higher concentration could potentially increase the detection of NS1 in urine and saliva by this kit. Development of a NS1 antigen detection test for use with such specimens would be a huge contribution to public health practitioners and would provide a powerful new tool for diagnosis of DENV infections.

6.C. METABOLOMICS-BASED DETECTION, PROFILING, AND IDENTIFICATION OF CANDIDATE SMBs IN SERUM, SALIVA, AND URINE ASSOCIATED WITH DEN AND SEVERE DEN INFECTION OUTCOMES.

6.C.1. Brief summary of results. The metabolomics studies were quite promising and provide proof of concept that this approach offers great potential for diagnosis and prognosis of DENV infections. An untargeted metabolomics LC-MS approach was optimized to identify compounds in acute phase clinical specimens (serum, saliva, and urine) to differentiate the DEN case disease outcomes, e.g. DHF/DSS, DF, and non-dengue (ND) disease using acute phase serum, urine, and saliva samples from Nicaraguan and Mexican patients. The approach identified many candidate compounds that reflected perturbation of the host metabolome by DENV infection, replication, pathogenesis, and the host response to the virus (Figures 22 - 29, Appendices 1-7). More candidate compounds remain to be identified by LC-MS/MS and will be added to this panel of metabolites.

The biochemical classes of these tentatively identified compounds included fatty acids and derivatives, amino acids, nucleosides, vitamin derivatives, phospholipids and sphingolipids (Tables 21 - 28). Tentatively identified (*in silico*) compounds in serum are provided in Figures 22 - 24, Tables 21- 24, in urine in Figures 26 and 27, Tables 25 and 26 and in saliva in Figures 28 and 29, Tables 27 and 28. The potential biological significance of compounds was determined in PubMed literature searches. Selected candidates from the panel of compounds identified in the untargeted (LC/MS) phase of this research that differentiated the DEN diagnosis groups (DHF/DSS, DF, and ND) and that were potentially biologically and physiologically relevant were selected and subjected to LC-MS/MS analysis to corroborate their identities. Six candidate SMBs or metabolites that differentiated disease outcomes were identified in serum specimens using purchased standards, including 1-oleoyl-lysophsphatidylcholine (lysoPC(18:1/0:0)) , 1-hexadecanoyl-sn-glycerol-3-phosphorylcholine (lysoPC(16:0/0:0)), 25-dihydroxyvitamin D3, proline, α-linolenic acid and docosahexaenoic acid. Two SMBs or metabolites, arginine and deoxyinosine were identified in urine using databases including METLIN and NIST (Figures 36 and 37). One SMB, xanthine, was identified in saliva using the NIST database (Figure 38). The potential role of each of these candidate SMBs as well as the potential pathway perturbed by DENV infection leading to the change in metabolite abundance were reviewed in Chapter 5.

Overall, the results obtained confirm metabolomic perturbations resulting from DENV infection and the immune response to infections of humans. For example, the disruptions found in SMBs related to lipid metabolism during DENV infections have been reported by others in *in vivo* and *in vitro* studies. [7, 8] Fatty acids and phospholipids were among the most perturbed SMBs probably reflecting their important roles in virus replication and endothelial cell homeostasis and endothelial barrier function.[289] These could be directly related to DENV pathogenesis and disease outcomes.

6.C.2. Future directions. The candidate SMBs identified that differentiate DEN disease outcomes provide the beginnings of a potential biosignature for DENV diagnosis and prognosis. Additional metabolomic profiling studies should be conducted to expand the panel of candidate SMBs. The panel of candidate SMBs will then need to be investigated for their diagnostic efficacy in clinical studies. Machine learning algorithms could be exploited to develop a robust metabolic biosignature that can be used by itself or integrated into existing clinical and laboratory algorithms for DEN diagnosis and prognosis. The preferred test schema to exploit the information and identified metabolites for diagnosis and prognosis in disease endemic areas will need to be selected.

Expanding the pool of candidate SMBs. The pool of candidate SMBs can undoubtedly be expanded by identifying by LC-MS/MS molecular features that have already been demonstrated in LC-MS analyses to differentiate the DEN diagnosis groups (Appendices 1 – 7). In addition, ongoing prospective studies in Managua, Nicaragua have resulted in collection of more than one hundred linked serum, saliva, and urine samples. LC-MS/MS analysis of these samples will hopefully validate already identified candidate SMBS and provide additional ones for characterization. Special emphasis should be devoted to discovery of additional candidate SMBs in saliva and urine samples. The extensive variability in metabolite abundance and prevalence in urine and saliva samples was problematic. The low FCs detected in metabolite abundances in the Nicaraguan samples as compared to the Mexican samples was of concern. LC-MS/MS analyses of the newly collected Nicaraguan samples should be a priority, and will likely be informative in terms of understanding potential variability in non-invasive samples and in generating additional candidate SMBs. A large pool of candidate SMBs for characterization in clinical studies is desirable.

Clinical studies to determine the diagnostic efficacy of the candidate SMBs. A critical next step is to determine the diagnostic and prognostic potential of candidate SMBs in prospective clinical studies in Nicaragua. These clinical studies should be expanded to include collection of samples from

predetermined time points post presentation in order to derive a “kinetic” metabolic profile through the time course of the disease. Comparison of the metabolic profiles of acute phase specimens (upon presentation) specimens collected near the time of defervescence (typically 3 to 4 days post presentation), and convalescent phase specimens would be most informative in terms of understanding fundamental mechanisms of DENV pathogenesis and in determining the utility of the respective candidate SMBs for predicting disease outcomes. Clinical studies should also address other epidemiological factors that could condition DEN disease and outcomes, including factors such as sex, age, infecting DENV serotype, primary vs. secondary infection, and clinical correlates of the metabolomics results. Clearly a major issue to be addressed will be inclusion of well characterized controls. The ND patients used as controls in these preliminary studies need to be replaced by patients with laboratory diagnosed infections. These should include patients who have viral and bacterial infections that are likely to be encountered in the diagnostic setting and could be clinically confused with DENV infections. For example, in Latin America there is a compelling need for differential diagnosis of DEN, influenza, and leptospirosis, to name a few. The panel of candidate SMBs as well as the molecular features that differentiate DEN disease groups (Appendices 1 - 7) should also be tested against other viruses that cause hemorrhagic disease in order to determine if the metabolites are also altered during similar immunopathologic and vascular diseases. It would also be informative to determine if the metabolites identified for DEN diagnosis and prognosis are perturbed in other flavivirus infections, such as yellow fever or West Nile fever. The latter viruses cause dramatically different diseases and different symptomatology. Metabolic profiling could potentially identify “flavivirus specific” perturbations of the metabolome and pathogenesis or disease and immune response determinants of metabolic changes. Overall such studies could provide insight into fundamental disease mechanisms and physiological pathways and processes involved. This could be of enormous value and could lead to development to therapeutic treatments for DEN.

The clinical studies should also consider whether or not to do a targeted metabolomics study. The metabolomic results presented in this research and results of previous studies [7, 56, 281] have consistently demonstrated perturbation of fatty acid and phospholipid metabolism during DENV infection. A number of metabolites from this biochemical class differentiated the DEN disease groups, demonstrating the critical importance of these pathways in DENV pathogenesis. Consideration should be given to focusing upon a lipidomics approach in the clinical studies. Optimization of sample preparation to target lipids and fatty acids, such as the use of methanol/chloroform and a column with optimal specifications for lipid detection, could result in a more specific metabolic biosignature of lipid biomarkers for DEN diagnosis and prognosis.

Machine learning algorithms. Future studies could implement machine learning algorithms and statistical models to identify a robust and focused metabolic biosignature for DEN diagnosis and prognosis. These studies would incorporate conventional clinical and laboratory test results and the identified candidate SMBs and extensive number of molecular features detected that remain to be identified by LC-MS/MS (Appendices 1 - 7). The machine learning function would identify the preferred test results and metabolites that may have been overlooked in this analysis to be incorporated in the metabolic biosignature for DEN prognosis. This approach could help reduce dramatically the number of statistically significant compounds to be identified by LC-MS/MS. Statistical programs are commercially and freely available for the implementation of predictive models using the metabolomics data. This could be a very fruitful area of research.

Metabolomics-based or POC tests for DEN diagnosis and prognosis. LC-MS/MS technology will not likely be applicable in the front line clinics where DEN patients first present for the foreseeable future. The long term goal of the research project is to select a subset of these pathogenically and physiologically relevant SMBs and then to determine the potential for the metabolite analyte or a surrogate (e.g., a protein involved in the metabolic pathway conditioning the metabolic change) to be

incorporated into diagnostic formats amenable to point of care tests (for example ELISA based formats). Alternatively, once the pathway from which the metabolite of interest is determined, enzymes in that pathway could be exploited for diagnosis. For example, a test to measure the enzyme activity could be developed to measure an enzyme that metabolizes phospholipids or fatty acids. A diagnostic algorithm incorporating results from such a POC test and conventional laboratory and clinical biomarkers could provide dramatically improved capability for diagnosis and prognosis of DENV infections and would be of immense value to physicians in managing patients.

However, it is also possible that in many of the larger urban areas a centralized metabolomics facility, for example in the Hideyo Noguchi research center in Merida or the "Hospital infantil Manuel Jesus Rivera", in Managua, could provide local or regional LC-MS/MS diagnostic capability for the clinics and other stakeholders in DEN diagnosis. Acute phase specimens collected in front line clinics could be aliquoted and a portion forwarded to the metabolomics facility. LC-MS/MS analysis could be provided in less than 24 hours (especially if performed in a targeted manner) and diagnosis and prognosis results provided to the attending physicians in the front line clinics the next day. Because of the typical 3 to 4 day time from presentation to defervescence and onset of vascular leakage in DHF/DSS patients, this would still permit targeting of patients destined for severe disease for clinical management. Dramatic advances in technology, including instrumentation and analysis software, are revolutionizing metabolomics, and this technology will undoubtedly become available in areas in the developing world. Certainly many centers, and indeed the two collaborating centers in Managua and Merida, already have the capacity to institute metabolomics centers. It is noteworthy in this regard that a number of the potential compounds that differentiated the DEN diagnosis groups were not identifiable by LC-MS/MS (Tables 21 - 28). Many of these may never be identified and thus will not be able to be incorporated into a POC, but they may end up being very important SMBs for DEN diagnosis and prognosis.

Nonetheless, centralized metabolomics facilities, as described, would be able to exploit this candidate SMBs. Perhaps both approaches will be employed for DEN diagnosis and prognosis in the future.

6.C.3. Lessons learned. Metabolomics is a very technologically demanding but robust new approach that offers great potential for diagnosis and prognosis of infectious diseases and for understanding fundamental pathogenic mechanisms that condition disease and disease outcomes. Much was learned about the adaptation and application of LC-MS/MS to identification of candidate SMBs for DEN diagnosis and prognosis. Some of the challenges encountered during the metabolomics analysis will be summarized briefly.

Reproducibility. HILIC columns can cause shifts in retention time during analysis of different batches of samples. For this reason, during statistical data analysis larger windows for retention time tolerance were allowed, which may have reduced the sensitivity and specificity of the analysis. The RT shifts could have caused false positive identification of molecular features associated with disease diagnosis groups, and artifacts or noise in the results. Guard columns had a negative impact on the reproducibility due to manufacturer defects, for this reason several analyses of non-invasive Mexican specimens were performed without guard columns.

Processing of samples. Sample preparation may cause loss of metabolites principally during protein precipitation with methanol and reconstitution of the sample in acetonitrile. There is no absolutely safe protocol to prevent metabolite loss during sample preparation. Future experiments should evaluate protocols that optimize the specimen preparation to achieve minimal metabolite loss, and also optimized for fatty acids and phospholipids detection, since these were relevant compounds identified in serum.

Controls. In this study, the negative controls were from febrile patients with unidentified diseases. Future studies should include as controls samples from patients with febrile illnesses of known etiology and with healthy individuals.

Contamination of specimens. The serum analyses were complicated because of the propensity of these specimens to be contaminated with polyethylene glycol (PEG), a polymer present in plastics, such as gloves, microcentrifuge tubes, and pipette tips. PEG contamination is introduced into serum either during collection, aliquoting and storage, or sample preparation. When present in high quantities, PEG will appear in the chromatogram as a series of peaks that differ from the next peak by a mass of 44 Da. This results in consecutive peaks with MW of 300, 344, 388, 432, 476, 520, 564, etc. PEG contamination was a common finding in serum specimens. When PEG is found at high concentration (visible in the chromatogram), the presence of PEG peaks could suppress detection of significant ions that otherwise would be high in abundance and therefore statistically significant. PEG ions were subtracted during data analysis by excluding the molecular weight (MW) of the ions during MFE, which avoids mistaking the PEG molecules as significant molecular features in the biological samples.

Inherent variability in non-invasive clinical specimens - urine. The inherent variability of urine and saliva samples made of analyses very challenging. Urine varies in an individual within a day due to the individual's hydration status, diet and climate temperature. Urine collection is a potential source of sample contamination if collection is not performed properly and following a standardized protocol. Inappropriate sample collection could introduce bacteria and synthetic contaminants. DEN diseased individuals are at potential risk to be dehydrated due to fever, anorexia, vomiting, and bleeding in severe cases. The use of urine specimens for disease profiling by comparison of groups of patients with diverse grades of hydration is challenging. Urine specimens for LC-MS analysis are typically normalized to creatinine or to osmolarity. In this research samples were normalized to creatinine, but the variability found during the analysis was considerable. Normalization to osmolarity should be investigated.

Inherent variability in non-invasive clinical specimens - saliva. Considerable variability was also detected in saliva samples. Saliva specimens present a different challenge than urine. Differences in the saliva specimens analyzed could have resulted from differences in mouth hygiene prior to sample

collection as well as the possibility of dietary differences, including ingesting drinks or food immediately before sampling. It is critical to optimize sample collection and preparation protocols. Sample collection can be standardized by the use of a saliva collection kit, which includes a neutral rinse prior to sample collection. Sample preparation needs to be optimized in order to remove as much variability in the samples as possible in order to obtain results that are easily reproducible among patients and among countries for successful biomarker identification.

Nonetheless a few candidate SMBs were identified in these acute phase clinical specimens and many more molecular features that statistically differentiated the diagnosis groups remain to be characterized. Thus there is potential for a metabolomics approach to predict progression to severe DEN disease in acute phase non-invasive specimens.

6.D. CONCLUSIONS

The DEN pandemic continues unabated across the tropical world and is increasing in public health importance. Approximately 30% of patients in Latin America now progress to severe DEN disease, which is overwhelming clinical care and public health capacity. This dissertation research is providing new information and tools to help address this globally important disease.

The commercially available NS1 antigen kit was adapted for mosquito-DENV surveillance in mosquitoes, and it proved to be a sensitive, rapid, and convenient test. NS1 is an excellent analyte for detection of DENV in *Ae. aegypti*. Implementing the test for DEN surveillance would be an important addition to the armamentarium for DEN control and would permit early intervention in and prevention of impending epidemics. The adaptation of the kit to detect DENV in non-invasive clinical samples was less successful. NS1 was detected in urine and saliva, but the test as constructed is not sensitive enough to use in DEN diagnosis. Nonetheless, it is possible that the test and sample preparation protocols could be optimized for each of the sample types. The adaptation of this technology to non-invasive clinical specimens would be of enormous benefit for DEN diagnosis.

The development of improved diagnostic and prognostic capability for DEN patients is critical. Metabolomics is providing an important new approach for investigating fundamental mechanisms of DENV pathogenesis and for differentiating DF and DHF/DSS in DEN acute phase specimens from DEN patients. The results of the LC-MS/MS analysis for differentiation of disease status were very promising. A panel of candidate SMBs that differentiate disease states is being developed. A metabolic biosignature for early diagnosis and prognosis of patients who will develop DHF/DSS would be invaluable. This "holy grail" of being able to predict DEN patient progression to severe DEN disease using acute phase clinical specimens may well be attainable. The application of metabolomics to prognose DENV infection outcomes has the potential to provide a true paradigm shift in DEN diagnosis and case management. In addition the application of metabolomics to reveal fundamental DENV pathogenic mechanisms will potentially provide exciting new targets for therapeutic interventions. Clearly metabolomics is destined to play a large role in helping to help address critical needs and opportunities associated with the DEN pandemic.

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APPENDICES

Appendix 1. Molecular features in Nicaraguan serum specimens that were statistically significant (based upon pairwise comparisons of molecular features using cut off values of P-value of $\leq$ 0.05 and a FC of $\geq$2, corrected P-value of $\leq$0.05) and that differentiated DEN disease states by HILIC LC-MS.

Mass	RT	Calculated formula	DHF/DSS-DF p-value	DHF/DSS-DF FC	DHF/DSS-NEG p-value	DHF/DSS-NEG FC	DF-NEG p-value	DF-NEG FC
110.1097	1.72	C8 H14			8.10E-04	-6.24	1.30E-03	-6.07
113.0842	1.92	C6 H11 N O			1.27E-09	16.20	1.19E-05	11.98
115.0635	16.09	C5H9NO2			6.65E-05	-8.88	5.37E-03	-6.74
127.0997	2.30	C7 H13 N O			1.20E-09	14.28	8.85E-06	10.36
128.0948	2.90	C6 H12 N2 O			1.06E-09	13.57	8.94E-06	9.91
133.0561	7.27	C5 H11 N O S			3.03E-02	4.95	2.35E-04	7.92
134.0948	1.82	C6 H14 O3			2.22E-06	8.34	1.04E-04	6.52
136.0383	6.21	C5H4N4O	2.58E-03	-7.16			1.01E-03	7.69
147.0174	1.44	C5 H9 N S2	1.79E-02	5.01	2.69E-06	8.39	7.33E-03	3.38
155.1308	1.80	C9 H17 N O			3.20E-09	11.56	5.48E-05	8.03
164.1049	2.07	C7H16O4			8.65E-03	-5.33	4.85E-03	-5.75
166.0394	1.49	C10 H7 Na O			4.18E-07	9.89	3.11E-04	6.61
168.0785	1.26	C9H12O3			1.09E-08	13.38	4.44E-07	11.64
169.1463	1.68	C10H19NO			1.02E-05	8.15	6.36E-03	4.51
176.0837	2.18	C11 H12 O2			1.02E-08	12.47	2.09E-06	10.34
183.1618	1.59	C11 H21 N O			1.64E-04	7.85	1.52E-02	5.05
196.1574	1.91	C11 H20 N2 O			1.20E-09	12.69	2.08E-06	10.14
205.0594	1.24	C8 H15 N O S2			4.73E-04	-6.94	1.22E-02	-5.31
208.149	1.25	C13 H20 O2	3.21E-03	-5.06	7.37E-03	-4.38	4.84E-02	0.67
214.1203	1.23	C11 H18 O4			9.84E-09	12.69	1.92E-06	10.76
222.1464	2.20	C10 H22 O5			3.79E-03	6.39	4.52E-02	4.79
225.1732	1.47	C13 H23 N O2	5.05E-02	-4.19	4.52E-05	-8.32	5.39E-02	-4.13
232.1308	1.23	C11 H20 O5			9.90E-09	12.67	1.91E-06	10.78
236.1621	2.02	C12 H28 S2			1.72E-02	5.17	8.84E-03	5.74
236.2251	10.64	C15 H28 N2			7.82E-06	8.18	2.33E-03	4.82
252.1127	1.49	C15 H17 Na O2			7.86E-08	12.19	1.97E-06	10.79
253.2397	1.44	C16 H31 N O	3.43E-02	-4.57	7.57E-03	-5.38		
255.1834	1.58	C14 H25 N O3	3.29E-02	4.49	5.41E-07	8.67	2.49E-03	4.18
258.1969	1.14	C16 H27 Na O	3.36E-04	6.76	7.36E-02	3.55	3.13E-02	-3.21
267.2041	2.19	C11 H26 N5 Na O			6.95E-03	6.00	1.23E-02	6.02
276.2087	1.11	C18H28O2	2.02E-05	8.38	2.93E-05	8.06		
278.2245	1.10	C18H30O2	1.20E-06	10.94	7.82E-06	10.01		

279.2558	1.40	C18H33NO	2.13E-02	-5.46	5.57E-03	-6.22		
283.287	1.39	C18H37NO			2.81E-02	5.30	1.07E-02	6.05
294.2196	1.10	C18H30O3	1.01E-04	8.66	1.00E-03	7.31		
295.2869	1.42	C19 H37 N O			1.29E-08	10.95	1.88E-06	9.64
296.1841	3.41	C14 H32 O2 S2			8.54E-06	8.11	3.63E-04	6.65
297.2667	1.47	C18H35NO2			9.77E-03	-4.60	7.63E-03	-5.20
299.2091	1.85	C15 H26 N5 Na			3.12E-05	7.31	9.69E-06	8.02
300.2088	1.16	C20H28O2	2.91E-03	7.30			8.43E-06	-9.55
302.2243	1.13	C20H30O2	7.49E-03	6.49	4.18E-02	4.98		
308.2199	1.99	C15 H32 O6			4.09E-03	5.97	1.79E-02	5.19
310.1989	3.04	C14 H30 O7			1.10E-05	9.42	1.67E-04	8.19
311.2304	2.61	C13 H30 N5 Na O2			3.19E-03	6.13	4.56E-02	4.57
312.23	1.19	C18H32O4	2.37E-03	6.93	8.65E-04	7.13		
313.2095	3.42	C12 H28 N5 Na O3			1.19E-05	7.21	4.44E-04	5.69
314.2235	1.15	C12 H31 N6 Na S	4.29E-02	-3.53	4.44E-08	-11.05	9.71E-04	-7.51
316.2396	1.09	C21H32O2			3.77E-07	-10.18	8.15E-05	-8.76
318.2209	1.22	C15 H31 Cl N4 O	1.18E-03	7.06			4.40E-03	-5.64
321.3023	1.40	C21 H39 N O			2.94E-05	6.72	9.15E-06	7.24
323.3184	1.37	C21 H41 N O			3.32E-06	9.01	2.51E-03	6.91
324.2145	2.74	C16 H36 O2 S2			1.05E-04	7.98	3.39E-03	6.39
325.2457	2.37	C19 H35 N O S			1.89E-03	6.63	1.16E-02	5.73
326.1938	4.50	C14 H30 O8			1.99E-04	7.59	1.23E-03	6.88
327.2253	3.02	C13 H30 N5 Na O3			3.36E-08	10.88	1.37E-07	10.83
328.238	1.09	C20 H33 Na O2	1.98E-04	7.44	8.39E-05	7.61		
338.2302	2.49	C16 H34 O7			3.12E-06	8.63	1.07E-03	6.19
343.22	4.50	C13 H30 N5 Na O4			1.07E-05	9.31	5.23E-04	7.86
346.1526	1.55	C19 H26 N2 S2			2.24E-09	-10.60	2.63E-08	-10.07
349.3334	1.38	C23H43NO			1.04E-08	10.96	1.88E-06	9.34
351.3481	1.42	C23 H45 N O			9.07E-08	10.49	8.15E-06	9.04
352.113	1.07	C16 H29 Cl S3			3.34E-03	5.94	1.46E-04	7.95
354.0642	1.05	C19 H12 Cl N4 Na	8.93E-03	5.12			1.07E-05	-7.51
355.2564	3.03	C15 H34 N5 Na O3			3.77E-04	7.07	1.00E-02	5.33
356.2911	1.17	C21H40O4			3.86E-04	7.09	6.32E-04	7.02
357.2358	4.09	C14 H32 N5 Na O4			1.75E-05	7.15	5.43E-03	4.23
359.96	1.37	C11 H4 O14	8.09E-02	4.61	7.34E-03	-4.53	2.61E-05	-9.15
364.3121	1.22	C27 H40	7.13E-02	4.23	1.97E-02	-4.04	3.13E-05	-8.27
366.3279	1.18	C27 H42	1.63E-03	6.98	1.03E-02	5.79		
368.3435	1.66	C27H44	2.89E-03	8.37	6.98E-04	9.33		
371.2517	3.60	C17H33N5O4			1.05E-08	10.95	1.26E-04	7.03
374.1702	2.32	C14 H29 Cl2 N6 Na			3.44E-02	4.04	4.45E-04	7.64
385.2669	3.18	C21 H39 N O3 S	7.07E-02	4.25	1.08E-08	10.59	3.17E-04	6.34

386.1757	1.23	C20 H26 N4 O2 S			3.88E-02	-4.63	1.63E-03	-6.85
387.2462	5.19	C16 H38 N5 Na S2			1.39E-04	8.28	7.67E-04	7.69
398.2428	1.38	C23 H35 Na O4			1.83E-02	3.16	2.20E-02	3.09
400.3312	1.25	C27H44O2	6.90E-02	4.27	6.30E-02	-3.76	9.91E-05	-8.03
411.2839	2.09	C13 H37 N11 O2 S			1.20E-05	7.25	1.23E-04	6.40
414.204	1.21	C24H30O6			6.12E-03	-5.32	5.22E-03	-4.55
415.2773	4.26	C18 H42 N5 Na S2	9.81E-02	3.93	1.09E-07	9.78	8.71E-04	5.85
416.3282	1.45	C27H44O3	5.33E-03	-5.03	7.35E-03	-4.96		
423.3549	1.52	C26 H49 N O S			4.07E-03	6.37	1.89E-02	5.33
428.2406	1.15	C22 H36 O8	4.21E-03	-7.08	2.07E-02	3.20	1.99E-06	10.28
429.2929	3.74	C19 H44 N5 Na S2	7.65E-02	4.29	8.18E-08	10.18	8.71E-04	5.89
430.3758	1.27	C20 H46 N8 O2	1.80E-04	-8.42			1.30E-02	4.98
431.2722	5.89	C18 H42 N5 Na O S2			9.66E-04	6.91	2.38E-04	7.97
444.3595	1.08	C29H48O3	7.06E-02	2.45	6.04E-04	-1.17	7.77E-03	-3.62
445.267	1.15	C23H35N5O4	5.41E-03	-6.33			5.83E-05	8.27
448.2072	2.88	C23 H41 Cl S3			8.88E-03	4.67	9.35E-05	8.09
455.3088	2.36	C21 H46 N5 Na S2			3.27E-07	8.49	9.68E-05	6.84
459.3034	4.96	C20 H46 N5 Na O S2			9.22E-06	7.80	8.84E-04	5.58
460.3505	1.07	C23 H45 N6 Na O2	7.02E-02	-3.50	5.20E-02	2.82	1.07E-04	6.33
467.3008	13.97	C25 H45 N3 O S2			2.63E-04	-7.38	8.78E-04	-7.07
473.3198	4.40	C21 H48 N5 Na O S2			2.03E-06	8.78	8.79E-04	5.73
475.2991	6.62	C21H41N5O7			2.42E-02	4.41	3.63E-03	5.81
477.2858	10.34	C24 H44 Cl N O6	9.51E-02	2.45	1.57E-03	-6.77	3.06E-06	-9.22
478.3663	1.07	C29H50O5			4.72E-03	-5.30	3.08E-03	-5.63
481.3169	13.86	C26 H39 N7 O2			2.62E-05	-7.82	8.05E-03	-5.67
483.3403	2.03	C23 H50 N5 Na S2			1.48E-05	6.78	8.78E-04	5.18
490.2843	0.86	C33 H39 Na O2	8.31E-02	3.66	4.77E-04	6.39	8.55E-02	2.73
494.3609	1.06	C30 H46 N4 O2			6.12E-04	-6.77	5.71E-03	-5.86
495.3329	13.67	C22 H46 N7 Na O2 S	9.29E-03	7.55	4.97E-03	7.95		
499.3358	2.70	C23 H50 N5 Na O S2			5.55E-08	9.08	9.69E-05	6.91
501.2853	10.05	C23 H48 N3 Na O S3	6.63E-02	3.12	4.26E-02	-4.26	1.29E-04	-7.38
506.4178	1.56	C25 H50 N10 O			2.78E-02	-4.35	1.12E-02	-5.11
509.3834	14.80	C29 H55 N3 S2	9.46E-03	5.88	2.45E-02	5.05		
509.4792	1.33	C32H63NO3			8.44E-03	-4.37	2.71E-02	-3.73
513.3508	2.48	C24 H51 N O10			6.16E-07	8.74	8.63E-04	5.66
517.3143	14.00	C26 H53 Cl N Na O S2			8.29E-03	6.34	1.01E-02	6.18
519.4282	0.57	C31 H54 N5 Na			8.71E-04	-3.61	1.00E-02	-3.12
521.348	13.74	C27 H52 Cl N O6	1.02E-03	9.16	4.03E-04	9.50		
527.3674	2.27	C25 H54 N5 Na O S2			3.26E-06	7.83	3.15E-04	6.07
529.28	9.23	C27 H44 Cl N O7			2.69E-03	-5.85	9.48E-03	-5.23
534.4486	1.54	C30 H62 O7			4.50E-03	-4.36	2.20E-02	-2.96

543.3626	3.08	C25 H54 N5 Na O2 S2			1.36E-06	7.83	3.09E-04	5.94
547.5298	1.42	C34 H70 N Na O2	5.01E-03	-5.31				
550.4438	1.69	C30 H55 N8 Na					2.57E-03	-5.46
551.4235	1.16	C38 H53 N3	2.59E-04	-7.51	4.23E-05	-8.04		
557.3147	13.96	C30 H48 N Na O5 S			4.94E-03	6.68	1.20E-02	6.09
567.4191	1.18	C38 H53 N3 O	1.89E-03	-6.96	3.21E-04	-7.43		
571.328	12.61	C27 H54 N3 Na O2 S3	2.10E-02	-4.70			7.90E-04	6.99
571.3922	2.57	C27 H58 N5 Na O2 S2			8.42E-06	7.55	8.63E-04	5.50
572.4784	1.07	C28 H61 Cl N10	4.54E-03	6.27				
573.3097	14.03	C30 H48 N Na O6 S			2.34E-03	6.59	1.97E-02	4.82
574.4938	1.15	C37H66O4	1.60E-02	-5.31	8.40E-03	-5.35		
579.3536	13.23	C24 H53 N9 O S3	1.88E-02	-4.47	4.19E-12	-12.28	4.54E-05	-7.81
584.2624	1.11	C33H36N4O6	2.29E-02	-4.93	1.01E-03	-6.67		
586.3006	1.13	C37 H43 N2 Na O S	1.14E-02	-5.42	6.03E-03	3.43	2.01E-06	8.85
588.4753	1.07	C37H64O5			5.38E-03	-4.59	3.87E-04	-5.44
591.3255	1.11	C40 H46 Cl N O			2.57E-05	5.89	2.38E-03	4.27
603.3249	1.12	C29 H49 N O12			2.31E-06	7.30	1.98E-06	9.31
613.7696	1.36	C11H9I3N2O4	4.87E-02	4.98			3.36E-03	-6.37
614.3299	1.12	C39 H42 N4 O3			8.22E-06	7.87	1.97E-06	9.80
627.1869	1.02	C37 H30 Cl N5 O S			6.15E-04	-4.84	1.96E-02	-3.19
630.796	1.38	C12 H2 N Na O20 S4			3.61E-05	-11.08	4.09E-05	-10.87
631.3566	1.12	C31 H53 N O12			1.21E-08	10.97	1.98E-06	10.49
633.641	2.04	C38 H79 N7			6.64E-03	-5.10	4.34E-02	-4.01
635.4143	12.79	C33 H62 N3 Na O3 S2			4.22E-03	-3.35	1.21E-02	-3.56
635.7503	1.46	C8 H2 Cl Na O22 S4	8.93E-02	4.21	2.53E-03	-6.68	1.92E-06	-10.89
642.3612	1.11	C33 H54 O12			6.21E-07	9.49	1.98E-06	10.07
645.6043	1.48	C40 H80 N Na O3	1.23E-03	-7.13			2.39E-04	8.09
645.7947	1.36	C9 H3 Na O26 S3			3.99E-02	-3.26	3.90E-04	-6.86
646.4514	1.26	C39 H63 N2 Na O2 S	3.46E-02	-4.89	5.90E-03	-6.00		
647.6205	1.71	C42H81NO3	2.04E-03	-6.91			5.61E-04	7.68
649.3669	1.16	C31 H55 N O13	2.29E-03	-6.64	3.48E-02	2.48	1.94E-06	9.12
651.7241	1.46	C8 H2 Cl Na O21 S5			4.00E-03	-5.93	5.35E-05	-7.77
659.3884	1.11	C33 H57 N O12			1.12E-08	11.59	1.97E-06	10.82
660.5493	1.29	C43 H73 Na O3			4.49E-04	-7.31	1.31E-05	-8.97
661.4211	12.61	C40 H61 Cl N Na O3			5.00E-03	-5.63	1.44E-03	-6.33
662.5596	1.38	C41 H78 N2 S2			6.40E-03	-5.27	7.60E-06	-8.53
668.5531	1.27	C47 H72 O2			4.33E-02	-3.44	1.85E-04	-7.13
670.568	1.47	C47H74O2			3.46E-08	-10.64	1.44E-05	-8.78
672.5202	14.37	C40 H72 N4 S2	5.28E-02	-4.17	1.66E-05	-8.76	3.87E-02	-4.58
677.5575	1.05	C36 H76 Cl N5 O4	1.71E-02	4.90			2.27E-04	-7.20
681.3565	1.05	C37 H55 N5 O S3			1.68E-04	-6.20	3.36E-03	-4.94

686.1935	1.18	C36 H35 N6 Na O S3	6.76E-02	-2.97	9.87E-04	-4.45		
687.419	1.10	C35H61NO12			2.59E-06	8.57	2.00E-06	9.79
692.5509	1.24	C42 H74 Cl N4 Na			5.18E-06	-8.55	3.46E-04	-7.05
699.549	2.32	C36 H78 N5 Na O2 S2			2.39E-02	-5.06	4.24E-03	-6.50
701.2051	1.02	C42 H33 Cl N3 Na O4			9.59E-04	-4.19	1.20E-02	-2.63
707.6204	1.70	C42 H82 Cl N5 O			1.14E-04	-8.01	1.16E-07	-10.97
709.6356	1.64	C43 H87 N3 S2					4.25E-03	-6.62
717.4575	12.25	C35 H72 N3 Na O4 S3	9.47E-02	-2.26	1.52E-05	-8.31	5.43E-03	-6.06
723.6134	1.49	C44 H88 Cl2 N Na	5.50E-02	3.15			1.12E-03	-6.09
726.8528	1.92	C22 H N O26 S	9.90E-02	3.69	2.21E-06	8.41	2.25E-03	4.71
731.6208	1.82	C44 H82 Cl N5 O			7.90E-03	-5.70	2.92E-04	-7.83
733.6351	1.56	C43 H88 Cl N O5			8.56E-10	-10.93	9.22E-08	-10.22
735.534	1.14	C48 H69 N3 O3	2.19E-02	-5.71			4.57E-04	8.29
735.5354	1.13	C51 H70 N Na O			1.31E-02	-5.62	2.07E-04	-8.59
742.9198	1.34	C19 H6 N5 Na O26			2.71E-07	-8.84	2.13E-10	-11.89
747.5191	8.54	C43H74NO7P			2.80E-03	-5.85	1.09E-03	-6.37
747.6144	1.24	C42H86NO7P			8.36E-03	-4.14	7.80E-04	-5.05
749.5224	1.12	C49 H73 Cl N Na O			2.50E-03	-5.65	1.08E-04	-7.27
753.5297	12.13	C29 H60 N23 Na			2.26E-03	-4.98	8.19E-02	-2.60
755.548	12.18	C39 H77 N7 O3 S2			6.94E-03	-6.65	1.71E-02	-5.97
756.3411	1.05	C53 H49 Na O S	6.53E-03	-4.75			9.87E-05	6.42
757.2136	1.27	C50 H32 Cl N3 O3			3.27E-04	-4.76	3.49E-02	-2.47
760.2125	1.28	C39 H41 N6 Na O S4	7.59E-02	-3.31	6.33E-04	-5.35		
763.5147	8.57	C38 H78 Cl N7 S3			3.65E-03	-6.55	1.64E-03	-7.06
763.5561	1.13	C44 H77 N O9			1.62E-03	-5.93	1.93E-05	-7.95
770.9133	1.45	C20 H6 N5 Na O27			3.87E-03	4.56	1.81E-03	5.23
771.5414	11.58	C40 H82 N3 Na O3 S3			2.49E-07	-11.14	1.49E-05	-10.10
775.224	1.02	C49 H31 Cl N7 Na			1.61E-03	-3.80	1.84E-02	-2.37
777.5311	11.87	C31 H60 N23 Na	9.06E-03	-5.57	1.13E-05	-8.18		
779.5478	11.88	C27 H61 N27 O			2.86E-06	-10.46	1.83E-06	-10.82
781.5627	12.03	C50 H80 N Na S2	6.82E-02	3.76	8.63E-04	-7.46	1.16E-07	-11.22
783.5787	12.26	C42 H86 N3 Na O2 S3	9.82E-02	3.92	1.32E-02	-5.92	2.28E-05	-9.84
789.5524	11.59	C43 H73 Cl N9 Na O	2.11E-02	-2.77	1.76E-04	-4.55	1.14E-04	-1.78
799.5714	12.06	C31 H66 N23 Na O			3.20E-04	-7.90	1.37E-05	-9.69
801.9023	1.24	C20 H15 N2 Na O25 S3	4.57E-02	3.14	2.35E-03	4.27		
805.5633	11.78	C35 H63 N23			1.24E-02	-5.72	3.95E-05	-8.82
807.5786	12.24	C43 H82 N3 Na O7 S			1.75E-03	-7.88	4.25E-02	-5.03
813.5517	11.98	C52 H71 N5 O3			6.50E-07	-11.02	9.16E-05	-8.72
814.8132	1.60	C19 H6 N Na O28 S3			1.92E-03	4.47	1.09E-05	7.57
815.567	11.50	C47H78NO8P	1.91E-02	-4.85	2.76E-08	-10.95	9.76E-04	-6.10
817.5824	11.63	C37 H77 Cl2 N15 O			7.58E-07	-10.10	1.57E-03	-6.45

819.5418	11.83	C35 H61 N23 O			1.15E-09	-12.98	3.96E-10	-13.45
821.5579	11.73	C37 H77 Cl2 N13 O3			2.18E-04	-8.38	1.54E-07	-12.48
823.5718	11.84	C48 H77 N3 O8	7.43E-02	4.89	2.51E-03	-6.97	1.28E-06	-11.86
824.6873	1.42	C53H92O6	1.36E-02	2.60	2.45E-02	-3.89	3.58E-05	-6.49
825.5869	12.01	C37 H76 Cl N17 S			1.46E-02	-5.36	1.14E-04	-9.29
826.7007	1.81	C53H94O6	4.61E-03	5.16			7.71E-04	-6.33
833.5939	12.40	C44 H75 N13 O S			1.07E-02	-7.52	1.28E-02	-7.58
837.5519	11.81	C41 H84 Cl N7 O2 S3			2.54E-05	-10.09	3.42E-06	-11.32
839.5653	11.27	C47 H82 Cl N O9			2.10E-04	-7.28	7.74E-04	-6.98
841.5815	11.35	C40 H76 N13 Na O3 S			2.18E-07	-10.54	2.56E-05	-9.12
845.5597	12.72	C47 H79 N3 O8 S			1.27E-02	-6.30	1.22E-02	-6.46
848.6875	1.18	C55H92O6	3.57E-03	5.79			1.67E-02	-4.39
849.5879	11.79	C45 H80 Cl N7 O6			1.05E-03	-7.72	8.77E-06	-10.48
850.7041	1.26	C55H94O6	2.56E-03	6.66			2.41E-04	-7.70
852.7204	1.84	C55H96O6	6.64E-02	4.27	1.33E-02	-4.85	4.91E-06	-9.12
856.9502	1.47	C24 H12 N5 Na O29			2.90E-04	6.45	2.00E-03	5.66
857.4688	11.71	C46 H65 Cl2 N11 O	4.55E-02	3.05	8.84E-05	-7.47	3.50E-08	-10.52
863.5598	11.81	C43 H87 Cl N3 Na O6 S2			4.30E-07	-9.82	1.17E-06	-9.79
865.5766	11.75	C49 H80 Cl N7 O2 S			3.78E-10	-12.67	1.18E-06	-10.57
872.6875	1.08	C57H92O6	4.18E-04	7.22			1.31E-05	-7.82
873.5066	1.08	C44 H76 N5 Na O7 S2	1.99E-02	-4.83	1.86E-03	3.81	2.08E-06	8.64
874.7047	1.35	C57H94O6	5.13E-02	3.53	4.79E-02	-4.34	9.50E-05	-7.87
876.719	1.27	C57H96O6	1.80E-02	5.39			1.75E-05	-8.58
878.7344	1.37	C57H98O6	3.55E-04	6.86	3.18E-02	4.53		
879.5576	11.72	C39 H81 N11 O5 S3			3.76E-04	-7.02	9.31E-04	-6.79
880.7504	2.40	C57H100O6	7.51E-04	7.11			4.17E-03	-5.73
887.5658	11.93	C45 H85 N5 O6 S3	8.66E-02	3.05	8.41E-02	-3.88	8.04E-04	-6.93
893.559	11.95	C52 H77 Cl N5 Na O4	7.47E-02	-3.85	3.54E-03	-5.79		
895.5751	12.40	C58 H82 N Na O S2			4.89E-03	-6.54	3.04E-02	-5.25
899.5378	11.72	C44 H84 Cl3 N5 O7	5.84E-02	2.93	4.37E-03	-6.12	7.29E-06	-9.05
900.7174	1.36	C51 H101 Cl N4 O4 S			1.10E-03	-6.55	8.02E-06	-8.43
916.7138	1.30	C51 H102 Cl2 N6 O S			5.19E-02	-3.71	1.39E-03	-5.72
937.0865	1.42	C17 H31 Cl3 N17 Na O21			3.33E-06	7.36	3.50E-05	7.10
951.105	1.33	C32 H21 N15 O21			2.99E-03	-6.46	9.80E-04	-7.88
961.6624	1.00	C58 H92 N5 Na O S2			3.42E-03	3.38	9.87E-05	5.28
975.9779	1.41	C32 H25 Na O30 S2			7.27E-03	-3.79	1.08E-02	-3.85
979.1335	1.33	C40 H37 N O26 S			1.01E-03	-5.31	6.50E-04	-5.68

Appendix 2. Molecular features in Mexican serum specimens that were statistically significant (based upon pairwise comparisons of molecular features using cut off values of P-value of ≤ 0.05 and a FC of ≥2, corrected P-value of ≤0.05) and that differentiated DEN disease states by HILIC LC-MS

Mass	RT	Calculated formula	DHF/DSS-DF p-value	DHF/DSS-DF FC	DHF/DSS-NEG p-value	DHF/DSS-NEG FC	DF-NEG p-value	DF-NEG FC
108.0941	1.43	C8 H12			6.06E-03	-6.68	3.46E-02	-4.62
110.1098	1.87	C8 H14			8.85E-05	-8.98	5.08E-04	-4.43
116.0839	2.14	C6H12O2			2.44E-02	-5.39	3.30E-03	-4.65
117.079	17.91	C5H11NO2			2.36E-04	-12.37	6.31E-06	-8.07
125.084	2.21	C7 H11 N O			5.34E-03	-6.63	1.17E-02	-5.47
126.0317	4.34	C6H6O3			9.61E-03	-6.27	2.53E-02	-5.50
133.0525	0.83	C8H7NO	6.87E-02	-3.27	6.04E-04	-7.76	7.15E-02	-4.68
138.1042	1.12	C9H14O			1.19E-02	-5.53	3.85E-03	-9.98
139.0995	1.91	C8 H13 N O			9.27E-04	-7.19	1.36E-02	-4.73
143.0945	18.79	C7H13NO2			9.05E-03	-7.92		
144.0422	4.36	C6H8O4			3.08E-03	-7.91	1.37E-02	-11.28
149.0508	9.26	C5H11NO2S			1.29E-02	-6.01	2.26E-02	-4.89
150.089	3.29	C6 H14 O4			4.87E-03	-8.43	1.94E-02	-5.17
152.0583	4.29	C7H8N2O2			5.64E-04	-9.46	2.41E-03	-4.92
154.1366	1.27	C10 H18 O			5.69E-04	-6.37	1.08E-03	-8.37
155.0346	17.79	C8 H6 N Na O			4.23E-03	-7.18	1.43E-04	1.96
156.1151	1.19	C9H16O2			1.38E-04	-7.93	1.21E-03	-8.80
162.0527	4.38	C6H10O5			1.22E-02	-6.25	2.79E-02	-14.01
162.1259	1.51	C8 H18 O3	5.29E-02	-3.94	1.59E-05	-10.07	1.02E-02	-5.02
164.1051	2.47	C7H16O4			1.82E-03	-6.49	1.31E-02	3.77
166.0992	1.35	C10H14O2			9.38E-03	-6.71	3.73E-02	-12.32
168.0785	1.32	C9H12O3			1.97E-02	-6.17	1.29E-02	-7.65
174.1402	1.12	C13 H18			3.33E-03	-6.09	1.56E-04	-8.81
178.0841	1.66	C7 H14 O5			1.93E-03	-6.03	6.44E-02	-4.50
180.0648	2.60	C6H12O6			2.90E-03	-9.53		
182.0708	1.66	C11 H11 Na O			1.73E-03	8.56	3.92E-02	-8.70
183.9781	30.71	C5 H9 Cl O S2					5.06E-03	4.87
184.1212	8.67	C9 H16 N2 O2			1.02E-04	-11.25	5.89E-04	-4.89
194.1153	4.00	C8 H18 O5			1.27E-04	-10.24	7.21E-03	-4.74
196.1209	2.45	C10 H16 N2 O2			3.12E-03	-7.34	1.77E-02	-7.96
197.0898	4.33	C6 H15 N O6			2.25E-03	-8.86	1.01E-02	-5.22
199.1574	1.25	C11 H21 N O2			1.48E-04	-9.40	2.18E-03	-10.36
202.0453	4.35	C6 H11 Na O6			1.36E-04	-11.73	4.13E-03	-5.23
202.172	1.12	C15 H22			2.97E-02	-4.88	3.90E-03	-8.46

208.0942	1.97	C8 H16 O6			3.34E-03	-6.44	2.33E-04	3.61
208.1312	3.39	C8 H17 N4 Na O			1.55E-05	-9.06	7.78E-04	5.42
210.1617	1.16	C13H22O2			5.62E-04	-6.62	2.62E-02	-7.15
211.1569	1.41	C12 H21 N O2			8.89E-04	-7.51	1.91E-02	-9.69
213.1724	1.27	C12 H23 N O2			9.69E-03	-6.11	5.07E-02	-9.36
215.1884	1.82	C12H25NO2			2.02E-04	-8.84	2.63E-04	-9.17
220.1673	1.57	C11 H24 O4			5.30E-04	-7.81	1.12E-04	-9.47
222.1463	2.80	C10 H22 O5			1.55E-03	-7.81	2.53E-03	-10.12
225.1729	1.24	C13 H23 N O2			8.98E-04	-7.82	3.21E-03	-8.03
226.1205	1.19	C12H18O4			3.27E-03	-6.77		
226.1682	7.65	C12 H22 N2 O2	7.77E-03	-4.74	3.64E-08	-12.40	5.90E-03	-9.37
227.1883	1.23	C13 H25 N O2			2.35E-04	-9.04	7.93E-03	-8.85
230.0708	1.10	C15 H11 Na O			7.58E-04	8.25	3.76E-03	-8.56
234.1621	1.13	C15 H22 O2	9.97E-02	-3.73	8.51E-03	-6.77		
238.1414	5.16	C10 H22 O6			1.32E-03	-9.21	4.18E-03	-9.28
239.173	3.95	C9 H22 N5 Na O			3.75E-03	-6.99	1.16E-02	-5.04
241.2405	1.44	C15 H31 N O			4.55E-03	3.65	7.65E-02	-6.93
243.1833	1.23	C13 H25 N O3			2.49E-03	-6.49	5.69E-03	-7.61
243.2565	13.26	C15 H33 N O	2.79E-02	4.87	2.39E-04	8.48	4.31E-02	-7.74
246.0852	2.68	C14H14O4			2.31E-03	-7.33	3.91E-03	-7.77
248.1986	1.36	C13 H28 O4			4.56E-03	-6.78	4.23E-02	-8.17
252.1571	4.46	C12 H20 N4 O2			2.87E-03	-7.04	9.14E-04	-9.20
253.2408	1.27	C16 H31 N O			8.78E-03	-7.05	3.02E-03	-8.46
254.1518	1.12	C12 H24 Cl N2 Na			3.04E-03	-6.50	9.40E-06	-8.15
258.1986	1.07	C18 H26 O			1.71E-04	-7.85	7.69E-08	-10.57
259.0718	4.30	C14 H10 N3 Na O			2.91E-03	-6.29	1.36E-02	-8.76
264.2458	1.27	C18H32O			4.90E-03	6.66	1.65E-02	-7.54
268.2194	1.14	C20 H28			2.24E-02	-3.41	5.08E-03	-7.86
275.0673	4.26	C7 H10 N9 Na S			6.44E-03	-6.84	4.67E-02	-5.46
278.2095	1.64	C14 H30 O5			5.04E-04	-8.61	3.32E-03	-8.18
280.0224	4.29	C13 H12 O3 S2			4.11E-04	-7.55	3.37E-03	-8.80
280.0442	1.08	C11 H20 S4	3.24E-02	-4.56	1.43E-02	-5.90		
282.1678	6.21	C12 H26 O7			1.88E-06	-11.77	8.57E-04	-9.62
282.1963	1.13	C20 H26 O			1.89E-04	-8.07	2.07E-04	-8.17
282.2555	1.07	C18H34O2			1.80E-02	-5.49	1.56E-02	-4.97
283.1994	5.17	C11 H26 N5 Na O2			2.57E-06	-11.13	1.78E-05	-8.97
283.2869	1.24	C18H37NO			3.09E-03	-8.50	4.45E-04	-5.09
292.2404	1.10	C19H32O2			3.39E-02	-5.35	3.56E-04	-7.25
294.1677	1.72	C13 H26 O7			9.58E-03	-6.70	3.98E-05	-9.26
294.2041	2.66	C14 H30 O6			1.19E-02	-6.54	2.16E-02	-6.85
294.2195	1.13	C18H30O3			9.12E-03	-5.04	7.92E-03	-10.13

296.1833	5.45	C13 H28 O7			3.39E-04	-8.12	2.24E-03	-8.01
298.192	1.15	C20H26O2	8.55E-02	2.34	1.19E-03	-7.78	8.24E-08	2.45
299.1945	6.21	C13 H25 N5 O3			7.02E-04	-7.21	1.13E-02	-8.21
299.2822	1.45	C18H37NO2			1.44E-03	-8.03	6.16E-03	-8.64
300.209	1.17	C20H28O2					5.73E-03	3.36
307.2513	1.25	C19 H33 N O2			7.26E-04	-8.08	4.85E-03	10.20
308.2195	2.30	C15 H32 O6			3.37E-04	-9.32	1.49E-02	-7.48
310.2152	1.12	C18H30O4			4.24E-03	-6.85	4.83E-02	-8.68
314.1268	1.18	C17 H18 N2 O4			2.02E-03	-7.37	1.81E-04	-8.42
316.2038	1.14	C20H28O3	8.75E-02	3.26			1.56E-03	-8.72
318.22	1.12	C20H30O3			2.52E-03	-8.02	2.77E-04	-11.10
322.1934	1.22	C22 H26 O2	4.68E-02	3.92			1.19E-04	-8.10
324.2145	3.95	C16 H36 O2 S2			1.89E-03	-8.14	5.64E-03	-7.69
324.2772	1.95	C19 H36 N2 O2			1.09E-05	-9.71	4.34E-05	-9.83
325.3715	12.74	C22 H47 N	6.71E-02	4.36	1.72E-03	7.89	9.83E-02	-6.90
326.194	7.31	C14 H23 N8 Na			3.18E-05	-8.99	2.93E-03	-7.57
326.224	1.12	C22H30O2	7.83E-02	3.37	2.91E-02	-5.83	4.91E-05	-8.90
330.2199	1.15	C21H30O3	8.76E-02	2.43	1.24E-02	-6.37	1.18E-05	-10.86
334.2145	1.12	C20H30O4			2.64E-06	-11.31	3.18E-06	-9.89
336.2145	1.37	C17 H36 O2 S2			8.73E-03	-6.04	9.57E-03	-9.20
338.1938	1.98	C15 H23 N8 Na			8.57E-03	-6.79	6.81E-04	-10.08
338.2303	3.29	C16 H34 O7			7.45E-03	-7.47	9.31E-03	-8.08
342.2191	1.13	C22H30O3					2.48E-03	-4.49
343.2204	7.31	C14 H26 N9 Na			7.60E-04	-7.38	2.35E-02	-7.43
344.1218	2.06	C14 H13 N10 Na			3.74E-02	-5.34	3.23E-03	-8.61
346.1965	4.00	C17H30O7			4.07E-04	-8.53	4.97E-04	-10.53
348.2301	1.18	C21H32O4			2.73E-03	-7.80	4.09E-04	-11.70
350.2098	1.11	C20H30O5			4.04E-05	-7.98	9.28E-06	-3.77
352.2098	1.81	C17 H36 O3 S2			1.36E-04	-8.85	1.74E-04	-8.64
352.2457	2.77	C18 H40 O2 S2			8.63E-03	-7.21	6.00E-02	-8.35
352.2462	2.17	C18 H40 O2 S2	7.94E-02	3.85	3.51E-03	7.02		
354.2168	1.21	C16 H32 Cl N4 Na O			6.57E-03	-7.00	6.99E-02	-8.40
354.2251	5.70	C16 H34 O8			5.07E-04	-8.28	1.59E-03	-7.94
355.2563	2.65	C15 H34 N5 Na O3	5.48E-02	2.02	1.62E-02	-5.13	9.17E-06	-8.67
356.2708	1.36	C24 H36 O2			7.55E-03	-6.64	3.81E-04	-10.80
357.3611	13.06	C22 H47 N O2	5.53E-02	4.44	2.58E-04	8.72	2.62E-02	10.07
360.1932	1.34	C21H28O5			2.56E-03	-6.73	6.22E-04	-10.33
362.2958	1.08	C25 H39 Na			2.62E-03	-6.36	7.54E-04	-6.32
364.2245	2.26	C21H32O5			2.79E-03	-6.49	1.01E-02	-8.29
366.2612	2.36	C18 H38 O7			3.49E-03	-7.49	3.88E-02	-7.98
368.2408	4.92	C17 H29 N8 Na			2.26E-06	-11.74	9.19E-06	-8.62

369.2723	2.33	C16 H36 N5 Na O3					5.18E-03	-7.23
370.2348	1.21	C22H30N2O3			4.41E-05	-7.85	1.03E-05	-10.06
376.3192	1.35	C21 H44 O5			4.40E-03	-7.11	1.83E-02	-6.58
380.2203	1.21	C16 H34 Cl2 N6			1.36E-02	-5.93	2.03E-04	-8.81
382.1087	4.38	C12 H23 Na O12			3.01E-04	-10.08	1.96E-04	-8.52
383.2522	1.95	C17 H37 N O8			2.34E-03	-6.49	9.32E-06	-6.68
385.2676	4.92	C17 H32 N9 Na			1.77E-05	-10.61	7.89E-05	-7.75
388.1038	1.04	C17H17ClN6O3			6.73E-03	-6.51	9.24E-02	
399.2827	4.16	C18 H34 N9 Na			8.36E-05	-9.70	7.36E-05	-8.09
400.3339	1.16	C27H44O2			2.16E-03	-7.64	1.25E-02	-10.72
410.2585	1.36	C20 H39 N2 Na O3 S	7.06E-03	6.82	7.21E-05	10.59	9.93E-02	-6.16
410.2874	2.46	C24 H42 O3 S			9.02E-03	-7.25	4.75E-04	-7.26
411.3183	2.08	C24 H45 N O2 S			3.17E-03	-6.29	9.28E-06	-7.87
413.2986	3.52	C18 H40 N5 Na O4			7.13E-03	-6.37	2.64E-02	-8.17
415.357	1.02	C26 H45 N3 O	5.55E-02	-4.00	3.08E-03	-7.40		
416.3275	1.31	C27H44O3			2.52E-04	-8.30	3.06E-04	-10.65
420.3452	1.50	C23 H48 O6			8.81E-03	-6.59	1.10E-02	-6.75
424.2682	1.59	C20 H33 N8 Na O			8.96E-05	-8.03	1.02E-06	-7.71
425.3326	1.81	C25 H47 N O2 S	6.27E-02	2.66			2.01E-04	-7.23
425.3506	13.06	C21 H48 N5 Na S	1.31E-02	5.94	2.42E-04	9.29	6.99E-02	-5.94
426.2538	1.16	C15 H36 Cl2 N10			4.21E-04	-6.56	3.57E-02	-7.84
426.2825	5.07	C25 H38 N4 S			1.52E-05	-10.85	1.10E-05	-8.17
429.2933	5.87	C19 H44 N5 Na S2			5.20E-04	-8.48	1.89E-03	-6.94
432.3227	1.73	C27H44O4			2.39E-03	-6.59	3.35E-02	-10.49
434.3606	1.49	C24 H50 O6			7.17E-03	-6.84	1.17E-02	-6.75
441.3444	1.27	C24 H44 N5 Na O			4.99E-05	-9.61	4.27E-05	-7.78
443.309	5.07	C25 H41 N5 S			1.54E-03	-8.39	1.09E-04	8.72
446.3965	1.27	C26 H54 O5			6.15E-03	-6.57	9.95E-03	-6.88
448.3762	1.46	C29 H52 O S			3.77E-02	-5.19	1.11E-02	-6.97
453.33	1.51	C22 H47 N O8			9.58E-03	-6.19	6.58E-03	8.49
457.3249	4.35	C21 H48 N5 Na S2			2.08E-03	-7.87	2.67E-04	-10.33
457.3407	1.44	C25 H47 N O6			3.02E-02	2.31	4.97E-02	6.50
457.4139	12.77	C28 H51 N5	5.90E-02	4.92	2.43E-04	9.78	2.61E-02	-5.81
464.3707	1.65	C26 H56 O2 S2			1.30E-02	-5.65	8.28E-03	-7.22
467.3011	14.89	C27 H42 N Na O4			8.62E-04	-9.03	1.23E-02	-6.73
468.3299	2.92	C24 H52 O4 S2			3.99E-04	-7.29	1.06E-03	-10.08
469.3248	1.33	C23 H49 Cl2 N3 O2			4.75E-04	-7.33	1.37E-04	-7.37
470.309	5.98	C27 H42 N4 O S			1.01E-04	-9.49	2.31E-04	-6.50
471.3038	2.69	C17 H42 Cl N9 O4			1.86E-03	-7.00	1.01E-05	-8.44
471.3405	2.88	C22 H50 N5 Na S2			7.86E-03	-7.07	4.32E-03	-7.79
473.2775	1.19	C28 H43 N O S2	4.95E-02	-4.28	1.85E-04	-9.17	7.17E-02	-5.86

473.3195	6.79	C21 H48 N5 Na O S2			1.10E-04	-9.24	6.57E-04	-6.73
477.3211	14.56	C27 H47 N3 S2	7.66E-02	-3.66	3.52E-03	-6.92		
479.3375	14.11	C29 H46 N Na O3			5.13E-04	-9.48	1.83E-02	-7.77
480.366	1.49	C25 H45 N8 Na			1.58E-02	-6.04	1.04E-04	-9.86
481.3166	14.72	C28 H44 N Na O4			2.32E-03	-7.94	2.73E-02	-7.37
482.3455	2.22	C26 H50 N4 S2			8.19E-03	-7.19	6.82E-02	-6.25
483.3409	1.83	C23 H42 N9 Na O			1.26E-04	-8.39	1.95E-05	-7.29
485.371	1.39	C19 H48 N11 Na S			9.05E-03	-6.73	1.75E-03	-7.06
490.4221	1.38	C28 H58 O6			6.73E-03	-6.32	4.13E-02	-8.21
492.378	1.09	C30H52O5			6.96E-04	-7.46	5.36E-03	-7.98
493.3165	14.68	C27 H39 N7 O2			3.16E-03	-8.41	1.02E-02	-6.98
495.3337	14.97	C23 H49 N3 O6 S			2.42E-04	11.69	1.22E-03	-9.14
497.3568	1.66	C24 H51 N O9			8.51E-03	-5.90	8.54E-03	-7.50
501.3508	5.20	C23 H44 N9 Na O2			5.60E-04	-8.57	8.80E-05	-7.22
501.3672	1.67	C27 H51 N O7			6.39E-04	10.14	6.35E-03	-6.45
507.3684	14.80	C27 H54 Cl N O5			2.17E-03	-8.35	1.34E-02	-6.65
509.3487	14.41	C30 H48 N Na O4			1.82E-03	-9.10	6.42E-03	-5.80
509.3633	1.04	C34 H48 N Na O			3.27E-03	-7.15	4.82E-04	-7.00
513.3869	2.54	C22 H47 N11 O3	5.47E-02	1.91	9.42E-03	-5.63	9.46E-06	-6.04
514.35	1.74	C20 H47 N10 Na O2 S			1.24E-02	-6.18	1.05E-02	-9.43
515.3661	3.56	C24 H54 N5 Na O S2			1.66E-04	-8.60	6.82E-04	-7.36
519.333	14.48	C21 H46 Cl N11 S			2.99E-03	-9.98	1.69E-02	-7.47
521.3493	14.80	C17 H40 N17 Na O			2.47E-04	9.60	1.24E-03	-8.11
523.365	14.72	C17 H42 N17 Na O			2.44E-04	10.67	5.71E-04	-7.81
525.3872	1.39	C26 H56 N5 Na S2			9.27E-04	-7.34	1.58E-03	-9.80
526.3717	2.60	C28 H54 N4 O S2			5.97E-02	-5.14	5.06E-03	-6.71
529.3822	3.09	C30 H51 N5 O S			4.86E-04	-8.84	3.87E-03	7.71
529.3975	1.56	C21 H52 N11 Na O S			2.16E-03	-7.58	5.52E-05	-7.60
534.4492	1.54	C29 H59 N4 Na O3			9.65E-03	-5.94	7.30E-03	-7.23
540.3867	2.30	C27 H57 N4 Na O S2			4.00E-03	-8.16	6.52E-02	-10.82
541.3835	1.87	C28 H57 Cl N3 Na O S			2.72E-03	-6.71	1.32E-04	-8.92
543.3324	14.29	C29 H50 Cl N O6			4.93E-03	-9.12	1.59E-02	-6.21
549.3784	14.13	C30 H60 Cl N O S2			4.78E-03	-7.37	2.53E-02	-7.89
550.4166	1.12	C29H61NO6P	4.66E-02	-3.80	2.90E-03	-7.02		
553.4551	1.79	C28 H60 N5 Na O4			1.10E-02	-6.61	1.72E-02	7.22
554.4028	2.07	C28 H58 O10			2.52E-03	-8.56	1.78E-02	-8.54
554.4033	1.90	C28 H59 N4 Na O S2			1.27E-03	8.50	7.14E-03	-7.30
555.3985	1.68	C23 H54 Cl N9 O4					2.76E-03	-7.45
559.3925	5.29	C31 H53 N5 O2 S			6.16E-04	-8.14	1.56E-03	-7.61
562.4604	0.98	C30 H63 Cl N4 O3			6.49E-04	-6.04	3.17E-02	-6.09
565.3149	14.68	C31 H48 Cl N O6			2.44E-04	9.62	8.92E-03	-6.82

571.4284	2.69	C32 H61 N O5 S					2.91E-03	-6.01
572.377	6.97	C40 H48 N2 O			4.81E-04	-7.83	2.31E-04	-5.62
573.4231	1.72	C28 H59 N7 O S2			1.87E-03	-7.40	1.73E-04	-11.32
578.4753	1.70	C32 H66 O8			7.11E-04	-7.06	6.09E-03	-6.07
579.3556	13.92	C28 H57 N3 O3 S3			8.86E-05	-8.60	8.54E-06	-8.12
581.4205	1.03	C21 H47 N19 O			8.81E-04	-8.42	5.18E-06	-7.33
584.4131	2.78	C37 H57 N2 Na S			5.47E-04	-8.87	1.16E-03	-5.88
585.3034	14.97	C15 H36 N19 Na O5			2.41E-04	10.43	8.69E-03	-7.25
589.4033	6.97	C27 H60 N5 Na O3 S2			5.54E-04	-8.05	4.13E-04	6.84
593.3697	13.82	C32 H55 N3 O3 S2			1.56E-04	-9.35	1.29E-03	-7.42
595.473	1.23	C37 H61 N3 O3			8.31E-04	7.81	1.55E-02	-6.63
596.4476	1.54	C36 H68 S3			3.03E-03	-6.50	4.06E-02	-6.64
599.4252	1.90	C33 H62 Cl N3 O2 S			5.43E-03	-6.41	6.43E-03	5.86
601.4397	2.78	C28 H60 N5 Na O7	9.72E-02	-2.92	1.25E-04	-9.85	1.15E-02	-6.59
603.4177	6.20	C24 H50 N15 Na O2			1.30E-04	-9.20	9.95E-05	-7.10
607.3882	13.65	C21 H46 N17 Na O3			5.99E-06	-8.68	1.04E-06	-8.83
609.3018	14.89	C29 H39 N9 O6			2.50E-04	9.23	8.87E-03	-7.04
611.3182	14.80	C21 H47 Cl2 N15 S			2.46E-04	9.17	8.82E-03	-7.99
617.4489	1.94	C33 H64 N5 Na S2			2.49E-03	-6.87	1.84E-03	5.61
626.4604	1.88	C32 H67 N4 Na O2 S2			2.16E-03	-8.25	1.13E-02	-6.80
629.4705	1.91	C29 H63 N11 S2	2.58E-02	5.20	8.24E-05	9.50	2.64E-02	-6.43
629.471	2.07	C30 H64 N5 Na O7			5.68E-03	-8.27	7.51E-02	-7.00
630.4846	0.97	C44 H63 Na O			2.42E-03	-6.39	4.78E-04	-6.31
631.4499	3.97	C30 H66 N5 Na O3 S2			9.84E-05	-9.06	4.80E-04	-6.18
633.4457	2.10	C33 H63 N O10			7.01E-03	7.67	4.35E-02	5.85
635.416	13.24	C28 H61 N9 O S3			6.81E-06	-10.20	7.31E-04	-7.06
640.4757	1.72	C38 H72 O S3			4.99E-03	-6.53	6.82E-03	-6.60
642.4546	2.81	C32 H67 N4 Na O3 S2			2.19E-03	-8.32	2.74E-02	6.24
642.5329	1.08	C41H70O5			3.07E-04	-6.82	1.33E-02	-8.66
643.4866	1.88	C32 H70 N5 Na O2 S2			2.19E-02	-6.55	1.28E-02	-5.66
644.5513	1.11	C41H72O5			4.74E-03	-6.56	7.62E-03	-3.97
645.4029	13.41	C39 H52 N5 Na O2			1.37E-06	-10.05	3.34E-09	-7.47
645.4661	3.36	C31 H68 N5 Na O3 S2			9.90E-03	-6.77	7.28E-03	-6.64
647.445	7.03	C35 H61 N5 O4 S			5.51E-04	-7.93	9.19E-06	-6.07
647.6207	1.30	C42H81NO3			2.35E-03	-7.10	1.99E-02	-4.47
649.4321	13.05	C31 H60 N7 Na O4 S			6.77E-03	-6.91	8.97E-03	-6.06
653.2906	14.97	C29 H43 N5 O12			2.42E-04	9.51	8.76E-03	-1.53
654.4913	1.57	C39 H74 O S3			5.02E-03	-5.75	3.61E-02	-6.61
656.4712	2.41	C41 H65 N2 Na O S			4.53E-03	-8.12	2.01E-02	-5.68
659.4168	13.34	C32 H66 N3 Na O3 S3			2.19E-06	-10.91	9.72E-11	-5.76
661.4602	6.31	C29 H63 N11 O2 S2			5.93E-04	-7.92	9.78E-06	-5.94

662.5625	1.10	C37 H78 N2 O5 S			5.99E-03	-6.52	7.18E-03	-6.81
663.4458	12.99	C36 H54 N11 Na			5.98E-06	-8.66	1.04E-06	-5.89
666.5365	1.06	C43H70O5			4.31E-04	-8.41	7.96E-05	-7.88
670.4717	1.04	C34 H67 N2 Na O9			2.48E-02	-5.39	8.08E-03	-5.77
670.4863	2.14	C42 H67 N2 Na O S			1.70E-03	-8.57	1.81E-02	-7.86
671.4823	1.76	C41 H66 N3 Na O S			7.50E-04	-7.17	1.76E-05	-5.36
672.5215	13.83	C40 H64 N8 O			2.52E-03	-7.87	3.53E-02	-6.55
675.4761	4.83	C37 H65 N5 O4 S			6.06E-04	-7.80	9.46E-05	-5.79
676.5436	1.07	C40 H73 Cl N4 O2			2.19E-05	-9.98	3.51E-06	5.91
682.4261	2.63	C44 H59 Cl N2 O2			4.84E-03	-6.71	1.66E-03	-5.71
682.5326	1.12	C42 H71 Cl N4 O			5.12E-02	-5.44	5.45E-03	-5.67
683.5543	2.26	C36 H78 N5 Na O S2			6.93E-03	-6.49	8.27E-03	-6.08
684.5019	1.93	C35 H73 N4 Na O3 S2			2.27E-03	-7.61	2.71E-02	-6.33
686.4814	3.50	C34 H71 N4 Na O4 S2			2.20E-03	-7.37	1.80E-03	-5.93
687.5124	2.15	C33 H70 N5 Na O8			4.66E-03	-8.39	4.03E-03	5.91
687.5141	1.95	C34 H74 N5 Na O3 S2			3.45E-04	8.57	8.19E-03	-7.25
689.4923	4.10	C33 H72 N5 Na O4 S2			7.65E-04	-8.34	1.17E-04	-6.39
699.4473	12.89	C39 H70 Cl N O3 S2			1.60E-06	-10.57	3.64E-09	-6.97
700.4971	2.93	C36 H76 O8 S2			2.57E-03	-7.91	5.19E-04	-6.93
702.5636	13.88	C40 H78 O9			4.11E-02	-6.22	1.34E-02	-6.06
703.5077	3.46	C32 H69 N11 O2 S2			1.00E-02	-6.94	2.16E-02	-6.11
714.513	2.49	C44 H71 N2 Na O2 S			3.84E-03	-8.04	1.33E-02	-5.53
715.5414	1.71	C30 H61 N21			1.05E-02	-5.30	6.89E-04	-6.12
717.5233	2.94	C34 H72 N5 Na O9			6.72E-04	-9.63	7.09E-03	-7.23
718.5531	1.09	C46 H79 Na S2			4.95E-06	-10.99	5.06E-05	-6.46
719.5017	6.38	C39 H77 N O4 S3			5.69E-04	-7.43	1.04E-04	-5.32
721.2792	14.97	C31 H47 N O18			2.44E-04	9.33	8.80E-03	-6.05
728.5825	13.77	C38 H81 N4 Na O5 S			9.25E-03	-8.04	1.33E-02	-5.15
731.5462	12.24	C37 H78 N3 Na O7 S			5.20E-03	-9.36	1.11E-03	-6.40
745.5549	2.20	C42 H75 N5 O4 S			1.81E-02	-6.75	1.02E-02	-5.96
749.3095	14.72	C32 H48 N5 Na O14	7.81E-02	4.00	2.46E-04	8.34	2.64E-02	-6.67
753.5306	11.89	C31 H59 N23			1.10E-02	-6.81	4.00E-02	6.74
755.5477	12.98	C42 H78 N5 Na O S2	8.73E-02	4.79	2.52E-04	10.51	1.61E-02	-5.93
757.5633	12.14	C31 H63 N23			5.89E-04	-13.91	4.40E-03	-7.67
759.5764	12.26	C29 H66 N23 Na			2.92E-02	-7.94	9.88E-03	-7.65
763.5513	11.73	C47 H69 N7 O2	7.81E-02	-3.56	6.81E-05	-9.63	1.53E-02	-5.22
765.5672	11.79	C41 H80 N3 Na O6 S			6.67E-03	-8.47	4.01E-03	-7.50
769.5966	12.37	C46 H85 Cl2 N O3			3.78E-03	-7.04	3.99E-02	-5.74
773.5581	12.28	C34 H71 N13 O7			1.28E-04	-12.73	2.52E-03	-8.75
775.5647	2.95	C37 H78 N5 Na O10			2.81E-03	-8.56	7.53E-03	-5.40
777.5327	11.78	C33 H59 N23			3.99E-03	-7.28	7.53E-03	6.59

779.5471	11.84	C33 H61 N23			2.07E-04	-10.22	6.97E-04	5.99
781.8172	11.81	C32 H2 N2 O13 S5	5.47E-02	2.56	3.11E-03	-8.55	1.02E-06	-6.01
782.0276	11.87	C54 H12 Cl Na O4			2.18E-03	-7.53	1.66E-03	-5.95
783.5769	11.99	C34 H74 N13 Na O6			1.01E-02	-9.87	3.65E-03	-6.13
789.266	14.97	C29 H47 N3 O22			2.44E-04	8.93	8.84E-03	-7.03
793.5983	11.92	C41 H87 N5 O3 S3			9.26E-04	-10.90	2.04E-03	5.62
795.5417	11.51	C48 H65 N11			1.44E-05	-10.48	4.71E-06	-5.02
797.5585	11.95	C51 H75 N O6			2.17E-03	-10.41	2.95E-03	-5.49
799.5724	12.09	C41 H84 Cl2 N7 Na S			1.59E-04	-11.55	4.93E-04	5.72
801.587	12.18	C53 H80 N Na O S			1.19E-02	-7.94	3.03E-02	-5.61
802.5646	3.62	C56 H75 Na O2			8.75E-04	-7.73	1.13E-04	-5.42
803.546	11.77	C50 H80 Cl2 N Na O			2.06E-03	-9.05	3.75E-03	-4.65
804.6139	13.24	C49 H80 N4 O5	1.58E-02	-3.32	5.87E-06	-8.62	2.01E-02	-5.39
805.5636	12.46	C40 H85 Cl N5 Na O3 S2			2.40E-04	11.26	2.50E-03	-9.04
807.5771	11.80	C57 H77 N S			4.50E-03	-9.73	1.31E-02	-4.42
808.1143	11.74	C53 H22 Cl N2 Na O4			5.81E-04	-6.58	9.13E-06	-5.47
809.5941	11.90	C37 H79 N9 O10			2.27E-02	-8.72	1.54E-02	-8.37
810.1999	11.84	C55 H31 Na O6			2.48E-03	-5.96	1.28E-03	-6.68
813.5543	12.27	C32 H68 Cl N21 O2	7.45E-02	3.42	2.00E-02	-6.94	4.05E-05	-6.27
815.569	11.68	C36 H66 N21 Na			3.67E-03	-8.02	1.27E-05	-5.65
819.5461	12.31	C44 H78 N Na O11	3.83E-02	-3.30	6.78E-05	-8.80	2.63E-02	-6.57
821.3853	1.02	C20 H52 N27 Na O2 S3			8.31E-03	-6.72	3.85E-02	-7.87
825.5889	12.51	C33 H68 N23 Na O			9.83E-04	8.79	1.50E-02	5.84
827.5983	11.96	C33 H73 N21 O2 S	8.59E-02	-3.65	1.37E-03	-8.74	8.08E-02	-6.88
829.5567	11.57	C32 H63 N25 O2			5.60E-03	-8.26	1.09E-02	-4.71
833.5936	12.37	C34 H73 Cl N21 Na			2.45E-04	9.79	4.74E-03	-5.60
835.6061	11.85	C34 H74 N19 Na O4			5.40E-03	-8.92	4.19E-03	-6.93
837.5527	12.25	C45 H80 Cl N5 O5 S			9.10E-08	-13.54	2.67E-08	-5.30
837.6172	11.98	C42 H91 N7 O S4			4.83E-04	-9.24	3.22E-06	-6.32
841.5825	11.48	C44 H88 N3 Na O4 S3			2.87E-03	-8.71	5.17E-03	-5.28
847.5713	11.74	C35 H66 N23 Na O			1.75E-02	-6.41	3.04E-02	-7.49
849.6019	5.23	C40 H87 N11 S4			6.97E-04	-8.69	4.81E-04	5.17
857.2541	14.96	C30 H48 N3 Na O24	4.66E-02	4.51	2.46E-04	8.63	2.67E-02	-6.66
863.5646	11.42	C47 H69 N13 O3			7.42E-06	-10.90	6.41E-08	-6.07
866.6971	1.07	C55 H95 Cl N2 O3			1.36E-05	-8.04	7.73E-08	-4.77
867.5954	11.49	C42 H82 Cl N13 S2			7.80E-05	-8.29	1.78E-05	-6.65
876.5788	1.03	C51 H80 N4 O6 S			8.94E-03	-6.09	2.22E-02	4.34
887.5615	11.38	C47 H77 N5 O11			2.50E-03	-6.73	9.32E-06	-5.38
891.6493	3.18	C48 H93 NO11 S			7.45E-04	-7.48	5.43E-04	-7.03
893.6284	6.04	C48 H87 N5 O8 S			1.78E-04	-9.02	1.87E-05	-5.29
921.6599	4.65	C51 H87 N9 O4 S			8.16E-04	-8.16	7.42E-05	4.30

924.6716	5.36	C52 H101 Na O5 S3			7.25E-04	-7.24	5.94E-04	4.12
937.6546	6.77	C57 H92 N3 Na O2 S2			9.06E-04	-7.61	3.60E-03	-4.29
948.6596	5.43	C51 H89 N8 Na O5 S			1.27E-04	-9.60	1.69E-06	-5.34
949.668	1.02	C55 H91 N5 O6 S			7.78E-03	-6.70	1.70E-02	-5.67
951.6703	6.11	C49 H101 N5 O4 S4			8.00E-04	-8.03	5.24E-04	-5.73
965.6865	5.43	C60 H91 N3 O7			5.66E-04	-8.70	3.98E-04	5.01
968.6975	6.10	C60 H96 N4 O2 S2			7.52E-03	-6.25	3.64E-04	-5.27
976.6492	14.67	C50 H97 Na O14 S			7.26E-04	-7.55	6.12E-02	4.28
978.6706	6.84	C55 H94 N8 O S3			4.80E-05	-8.94	1.11E-03	-4.18
979.7015	4.73	C54 H93 N9 O5 S			8.21E-04	-7.80	7.45E-05	-5.42
988.6488	14.63	C40 H72 N30 O			2.34E-04	-9.29	6.85E-03	-6.28
991.2153	14.55	C18 H50 N17 Na O21 S4	7.88E-02	-2.98	5.23E-04	-7.87	5.58E-02	-7.21

Appendix 3. Molecular features in Mexican serum specimens that were statistically significant (based upon pairwise comparisons of molecular features using cut off values of P-value of ≤ 0.05 and a FC of ≥2, corrected P-value of ≤0.15) and that differentiated DEN disease states by RP LC-MS analysis.

Mass	RT	Calculated formula	DHF/DSS-DF p-value	DHF/DSS-DF FC	DHF/DSS-NEG p-value	DHF/DSS-NEG FC	DF-NEG p-value	DF-NEG FC
132.0721	7.71	C5 H12 N2 S	3.35E-04	5.71	1.39E-03	5.93		
151.0634	4.00	C8H9NO2			5.70E-03	7.89	5.09E-04	9.32
156.0279	1.23	C4 H12 O2 S2	4.52E-03	-5.28	5.23E-03	-5.51		
175.0632	9.24	C10H9NO2	1.07E-03	-5.23	2.10E-02	-4.69		
176.0949	2.08	C10H12N2O	1.01E-04	-6.30			1.32E-02	3.99
181.0739	3.34	C9H11NO3			1.60E-02	5.16	2.59E-03	6.40
182.0706	9.07	C11 H11 Na O			7.22E-04	-6.48	3.97E-02	-4.12
188.1164	1.01	C8H16N2O3			6.92E-03	5.00	4.94E-03	5.11
189.079	9.86	C11H11NO2	1.89E-04	-6.60	1.23E-03	-6.64		
201.0084	8.84	C10 H4 Cl N3	2.66E-04	6.03	1.73E-05	7.65		
202.1317	1.77	C9H18N2O3	1.27E-07	-8.98	7.81E-05	-8.36		
207.0571	1.22	C9 H15 Cl2 N	4.98E-02	-3.47	2.68E-03	-5.42		
218.1263	3.01	C9H18N2O4	1.51E-03	-5.07	1.72E-04	-6.51		
225.0747	1.03	C14H11NO2			9.26E-04	6.55	1.37E-06	7.99
230.1632	4.00	C11H22N2O3	7.87E-04	-6.34				
232.1426	1.29	C10H20N2O4	6.59E-03	-5.07	3.22E-03	-6.02		
262.0506	8.84	C9 H19 Na O S3	2.60E-03	5.14	1.60E-04	6.96		
266.1266	2.22	C13H18N2O4	4.61E-03	-4.51	4.36E-05	-7.35		
278.1632	8.17	C15H22N2O3	2.65E-04	-5.36	6.01E-04	-6.24	5.02E-02	-0.89
285.0894	1.16	C10 H15 N5 O3 S	8.93E-03	-4.94	1.76E-02	-5.06		
294.1216	3.03	C14H18N2O5	1.44E-02	-4.08	5.74E-06	-8.56	3.59E-02	-4.48
298.1335	13.00	C20 H19 Na O	1.69E-02	4.51	9.79E-04	6.64		
304.1988	0.93	C14 H28 N2 O5	2.14E-02	4.11	7.02E-03	5.30		
312.0777	7.23	C13 H16 N2 O5 S			7.78E-03	5.81	5.34E-03	6.03
325.1746	1.43	C14H23N5O4	1.19E-03	-5.34	3.55E-03	-5.18		
327.0953	1.96	C14H17NO8			8.33E-03	6.96	2.30E-03	7.92
331.2107	8.13	C15H29N3O5	1.67E-02	-4.62	4.31E-03	-5.95		
333.1323	6.31	C16H19N3O5	1.50E-04	-7.01	8.00E-03	-5.25		
341.1486	3.61	C17H19N5O3	6.70E-05	-7.68	9.55E-05	-8.65		
351.1585	8.53	C20H21N3O3	6.76E-04	-6.18	1.67E-04	-7.98		
357.3606	13.40	C22 H47 N O2	1.66E-02	4.13	1.49E-03	5.85		
365.0468	1.96	C17 H11 N5 O S2			4.11E-02	4.68	1.66E-03	6.98
371.3029	15.44	C21 H42 Cl N3	1.46E-03	5.61	3.25E-03	5.98		

381.0146	1.97	C20 H7 N5 S2			3.51E-02	4.48	1.25E-03	6.88
403.8833	8.21	C14 H2 Cl Na O7 S2			1.65E-02	-5.04	2.53E-03	-6.14
410.2582	16.78	C20 H39 N2 Na O3 S	1.48E-02	5.71	1.51E-03	7.99		
413.2927	10.04	C26 H39 N O3	2.01E-03	5.47			4.78E-02	-3.73
414.204	11.82	C24H30O6			1.83E-03	6.30		
431.3031	10.27	C26 H41 N O4	6.14E-04	6.51	4.36E-02	4.24		
454.1562	8.32	C24 H26 N2 O5 S			9.54E-04	-6.46	1.06E-02	-4.99
463.2755	10.68	C26 H41 N O4 S	6.77E-04	6.53	4.60E-03	6.22		
467.3012	12.03	C18 H37 N13 S	1.44E-02	-0.76	6.57E-03	-1.00		
479.2705	9.89	C26 H41 N O5 S	5.04E-03	5.06	2.37E-02	4.78		
479.3364	13.45	C27 H49 N3 S2	6.97E-03	-3.44	3.58E-02	-3.26		
504.2774	16.81	C26 H48 O3 S3	1.24E-02	4.67	2.43E-03	6.20		
509.2957	7.45	C31 H43 N O3 S	1.28E-04	-6.83	2.61E-03	-6.42		
511.3271	10.65	C29 H46 N Na O5	5.34E-02	-2.90	4.28E-03	-4.59		
511.378	16.98	C26 H54 N3 Na O3 S	1.88E-03	5.97	2.74E-03	6.53		
524.3735	16.80	C34 H49 N2 Na O	1.31E-02	5.11	2.58E-03	6.80		
528.399	15.62	C29 H57 Cl N4 S			2.22E-04	-6.75	3.35E-02	-4.31
534.248	12.21	C29 H34 N4 O6	1.57E-02	4.05	6.59E-03	5.03		
537.3793	15.05	C30 H55 N3 O S2	4.38E-03	-4.13	4.82E-03	-4.66		
539.3574	11.41	C35 H50 N Na S	6.23E-02	-3.31	3.07E-04	-7.08	5.80E-02	-3.78
551.3944	15.81	C30 H53 N3 O6	7.37E-03	-2.83	4.07E-02	-2.62		
561.3129	14.11	C35 H47 N O S2	1.51E-02	-4.29	2.15E-03	-5.91		
562.2465	12.03	C24 H41 Cl3 N8 O	1.83E-02	-4.32	8.45E-04	-6.58		
563.2678	16.81	C31 H46 Cl N O2 S2	1.26E-02	4.65	1.66E-04	7.26	5.45E-02	2.61
566.8817	13.69	C20 H2 N Na O14 S2	2.77E-03	-5.33	3.40E-03	-5.65		
567.4409	16.79	C35 H57 N3 O3	1.20E-02	5.42	1.33E-03	7.49		
569.3472	13.29	C35 H48 N Na O4	1.29E-02	3.33			2.80E-02	-3.51
573.2868	1.13	C30 H43 N3 O6 S			2.15E-03	-6.52		
589.4467	16.94	C35 H63 N3 S2	1.52E-03	-5.48	4.10E-03	-5.29		
590.6171	8.46	C40H78O2	2.18E-02	-4.42	1.67E-03	-6.67		
604.2939	13.71	C27 H55 Cl3 N4 S2	1.98E-02	-3.82	9.94E-04	-6.05		
627.2652	12.89	C40 H38 Cl N3 O2	2.85E-02	-3.85	1.55E-04	-7.16		
667.3661	7.48	C29 H50 N13 Na S2	5.93E-03	-5.18	5.16E-03	-5.58		
748.3751	8.03	C32H60O19			8.84E-04	-7.46	4.03E-02	-4.59
768.4034	8.50	C22 H53 N18 Na O11	1.11E-02	-3.37	1.10E-02	-3.81		
773.556	16.04	C37 H75 N9 O6 S			2.38E-02	-5.77	7.75E-04	-7.91
786.3248	8.04	C40 H47 N10 Na O2 S2			3.94E-03	-4.93	9.79E-03	-4.51
823.2979	7.47	C33 H46 N13 Na O7 S2	6.67E-02	-3.45	1.61E-03	-6.41		
828.3761	7.74	C44 H60 O13 S	5.80E-02	-3.28	1.32E-03	-5.63		
863.4023	8.35	C46 H61 N3 O11 S			2.02E-03	-6.46	2.79E-02	-4.62

899.4712	7.63	C40 H70 N5 Na O16	2.37E-03	-5.52				
904.476	7.46	C35 H69 N12 Na O12 S			7.24E-04	-7.48	3.47E-02	-4.56
908.133	8.26	C18 H29 N16 Na O26			4.41E-04	-6.84	1.42E-02	-4.92
918.4959	9.51	C36 H58 N26 O2 S	1.05E-03	-5.90			1.43E-02	4.98
941.462	8.30	C45 H68 N5 Na O15	1.04E-02	-2.64	2.02E-02	-2.93		
973.6535	12.96	C50 H83 N15 O3 S	4.27E-02	-3.24	1.21E-03	-6.04		

Appendix 4. Molecular features in Nicaraguan urine specimens that were statistically significant (based upon pairwise comparisons of molecular features using cut off values of P-value of $\leq$ 0.05 and a FC of $\geq$1,) and that differentiated DEN disease states by HILIC LC-MS analysis.

Mass	RT	Calculated formula	DHF/DSS-DF p-value	DHF/DSS-DF FC	DHF/DSS-NEG p-value	DHF/DSS-NEG FC	DF-NEG p-value	DF-NEG FC
107.0399	8.33	C3H6FNO2	4.27E-05	-1.37	5.20E-04	-1.12		
111.0433	29.55	C4H5N3O					1.93E-03	1.06
115.0614	3.02	C5 H9 N O2	2.19E-04	-2.29	1.49E-04	-2.40		
117.0789	15.39	C5H11NO2	5.04E-05	-1.25	1.23E-07	-1.48		
119.0586	7.26	C4H9NO3	1.68E-03	-2.45	5.76E-04	-2.78		
123.0792	3.01	C6 H9 N3	1.38E-03	-1.21	1.95E-07	-2.59	1.30E-03	-1.38
130.0425	1.19	C9 H6 O			2.30E-04	-2.08	1.15E-03	-1.05
130.1107	19.82	C6H14N2O	8.06E-04	-1.79	9.19E-04	-1.88		
131.0784	5.53	C3 H9 N5 O	2.46E-03	-1.60	2.18E-07	-3.05	1.96E-02	-1.45
136.0372	5.37	C4 H9 Cl N2 O	4.09E-03	-1.76				
137.0475	16.48	C7H7NO2			3.06E-04	-3.39	3.48E-05	-1.94
143.074	5.10	C10 H9 N			1.08E-05	-1.43	7.92E-05	-1.17
143.0946	15.60	C7H13NO2	6.45E-04	-1.23	7.42E-04	-1.46		
146.0454	5.23	C3 H6 N4 O3	8.73E-03	-1.42	9.31E-08	-3.24	1.74E-03	-1.81
147.0566	2.10	C3 H9 N5 S	9.77E-03	-1.27	6.32E-04	-1.74		
149.0508	8.30	C5H11NO2S	3.36E-04	-1.61	3.85E-03	-1.20		
151.0299	15.60	C4 H9 N O3 S			9.60E-05	-1.63	1.58E-06	-1.33
151.0637	1.10	C8H9NO2	2.15E-04	1.49	1.43E-06	2.38		
153.0532	4.21	C6H7N3O2	1.45E-03	-1.44	1.44E-04	-1.91		
157.0716	2.75	C7 H11 N O3	4.37E-03	-1.35	1.39E-03	-1.56		
159.1259	17.83	C8H17NO2			1.59E-06	-2.48	3.31E-11	-1.63
165.0652	9.58	C6H7N5O			2.34E-03	-1.11		
169.0532	1.24	C11 H7 N O			2.61E-03	-1.43		
172.1208	6.84	C8 H16 N2 O2	8.30E-05	-1.73	2.22E-05	-2.04		
175.0035	16.38	C4 H5 N3 O3 S			9.98E-03	-1.84	1.16E-03	-1.48
178.099	1.11	C11H14O2			2.27E-06	-1.38		
179.0806	6.93	C7 H17 N S2	1.96E-06	-2.30	4.20E-05	-2.14		
181.0505	16.90	C5H12NO4P			6.81E-04	1.43	3.45E-04	1.53
181.0597	6.49	C6 H7 N5 O2			6.03E-03	1.03		
181.0742	5.26	C9H11NO3			1.91E-03	-1.30	1.83E-03	1.11
182.1052	6.65	C9 H14 N2 O2	7.60E-03	-1.23	4.43E-03	-1.43		
187.0634	5.03	C11 H9 N O2					1.61E-03	-1.03
188.9861	15.61	C3 H8 Cl N O4 S			5.11E-06	-1.08		
190.0954	16.31	C7H14N2O4			4.13E-04	-1.52		

191.058	5.31	C7H13NO3S					6.03E-08	1.56
194.1031	6.89	C6 H10 N8	2.93E-05	-2.15			7.03E-05	2.34
199.0294	3.06	C8 H9 N O3 S					4.62E-04	1.27
201.1096	13.49	C13H15NO	7.94E-03	-1.01				
202.0452	3.95	C12 H10 O S			2.09E-04	2.58	1.47E-03	2.73
202.132	17.77	C9H18N2O3			1.46E-06	-1.65		
203.0823	1.31	C8 H14 Cl N3 O					4.83E-11	1.17
204.0896	10.61	C11H12N2O2			4.04E-03	-1.38		
204.1112	22.72	C8H16N2O4	4.99E-03	-1.07	3.84E-03	-1.30		
204.1471	28.34	C9 H20 N2 O3	8.24E-04	-1.14	5.63E-06	-1.62		
208.0848	5.29	C10H12N2O3					6.46E-04	1.03
210.0652	2.80	C9H10N2O4			4.10E-04	-1.97	1.77E-07	-2.43
211.0862	5.39	C10 H14 Cl N3			1.45E-03	-1.06		
213.0463	2.30	C9 H11 N O3 S	5.44E-03	-1.24	5.26E-03	-1.27		
213.0472	1.49	C6 H15 N O3 S2	1.89E-04	-1.44	5.08E-05	-1.40		
214.1316	17.40	C10H18N2O3	1.40E-03	-1.19				
215.056	16.72	C5H14NO6P	7.59E-05	-1.52				
216.1102	2.98	C9H16N2O4					1.30E-02	1.13
216.1475	19.12	C10H20N2O3	2.09E-04	-1.33				
217.1414	5.59	C9 H19 N3 O3			3.29E-03	-1.14		
218.1264	22.50	C9H18N2O4			6.81E-03	-1.03		
219.1288	5.00	C6 H17 N7 S	6.48E-06	-2.11	1.46E-03	-1.34		
224.0797	5.24	C15H12O2					2.15E-05	1.45
225.0749	10.05	C14H11NO2					1.36E-07	3.32
229.1791	22.32	C11 H23 N3 O2	5.12E-03	-1.29	5.28E-03	-1.31		
230.1229	4.34	C6 H14 N8 O2	2.11E-03	-2.20	4.93E-03	-1.97		
231.1202	5.44	C9 H17 N3 O4	3.08E-04	-1.93	8.60E-03	-1.41		
231.1577	5.54	C10H21N3O3			4.40E-03	-1.28		
232.1025	4.19	C13 H16 N2 S			3.59E-03	-1.32		
234.0771	13.52	C12 H14 N2 O S	2.07E-04	-1.01	1.87E-03	-1.00		
237.085	15.82	C8 H15 N O7			3.31E-04	-1.09		
239.1021	9.26	C9H13N5O3	2.95E-03	-1.27				
240.0878	17.66	C9 H20 O3 S2			2.75E-06	-2.14	3.22E-06	-1.20
240.1476	18.49	C12 H20 N2 O3	2.13E-03	-1.18				
242.1631	21.11	C12 H22 N2 O3			6.26E-03	1.12		
243.0718	6.06	C14 H13 N O S			4.68E-05	1.23	8.61E-03	1.16
245.0832	7.14	C9H12FN3O4	2.04E-04	-1.40			5.53E-06	1.69
245.1014	18.66	C9 H15 N3 O5			2.81E-03	-1.60		
250.957	5.79	C8 H7 Cl2 N O2 S	3.10E-10	-1.03				
251.0998	16.98	C10H13N5O3			3.40E-05	-1.07		
252.1377	1.16	C9 H21 Cl N4 O2					7.89E-05	-1.06

254.0399	18.86	C11H10O7			1.98E-08	-1.03		
255.0681	5.40	C10 H13 N3 O3 S	1.86E-06	-1.15	3.42E-07	-1.27	1.99E-03	1.80
255.0971	10.29	C15H13NO3	4.27E-04	-1.51			2.33E-07	1.55
256.1819	1.09	C13 H24 N2 O3			6.22E-03	-1.10	2.43E-04	-1.31
260.1013	15.80	C10 H16 N2 O6					9.40E-04	1.25
260.1217	4.11	C11 H20 N2 O3 S	2.62E-06	-2.56				
262.0766	1.08	C21 H10	9.28E-04	-1.39	2.82E-04	-1.29		
264.0895	0.94	C16 H12 N2 O2	3.38E-03	-1.42				
266.103	18.33	C11 H22 O3 S2			8.22E-03	1.27		
267.0967	6.89	C10H13N5O4	2.02E-04	-1.64				
268.0804	5.32	C10H12N4O5	2.03E-07	-1.81				
268.996	0.85	C9 H7 N3 O3 S2	3.20E-04	1.01	1.85E-06	1.81		
269.2064	7.51	C10 H23 N9	1.89E-06	-2.08	3.39E-06	-2.22		
270.0678	2.72	C19 H10 O2					1.03E-03	-1.22
273.0872	18.55	C9 H15 N5 O3 S	3.44E-05	-1.29	9.11E-05	-1.20		
278.1034	18.56	C9 H18 N4 O4 S			3.89E-03	-1.39		
280.0892	4.34	C12 H21 Cl O3 S	4.32E-03	-1.73	6.04E-04	-2.22		
283.1769	5.40	C11 H21 N7 O2	2.02E-03	-1.56	8.19E-04	-1.77		
285.0957	4.75	C11 H15 N3 O6	2.82E-03	-1.20				
285.1324	19.05	C12H19N3O5	2.09E-07	-3.37	2.81E-16	-5.55	6.61E-04	-2.18
285.1929	10.91	C15 H27 N O4	1.70E-03	-2.69	1.11E-03	-2.74		
286.1525	3.09	C17 H22 N2 S	8.51E-04	-1.93				
287.112	15.02	C11H17N3O6	2.25E-05	-1.16	1.57E-05	-1.32		
292.0825	16.45	C9H16N4O5S1			6.46E-03	-1.73	1.05E-03	-1.24
292.11	2.74	C11 H21 Cl N4 O S	4.79E-07	-1.09			6.61E-02	1.03
293.0438	10.85	C7 H11 N5 O6 S			5.89E-03	-1.50	3.49E-03	-1.21
295.0565	10.59	C8H14N3O7P			4.84E-06	1.79	7.12E-04	1.09
297.1078	6.29	C11H15N5O5	5.98E-06	-1.91	1.28E-03	-1.43	1.51E-02	-1.07
297.1804	7.53	C9 H19 N11 O	2.49E-03	-1.81			5.60E-04	1.57
297.1924	5.20	C12 H23 N7 O2	7.55E-08	-1.63	4.74E-08	-1.77		
299.2096	10.64	C12 H25 N7 O2	1.33E-06	-2.19	8.24E-06	-2.60		
301.1893	5.45	C16 H31 N S2	1.26E-03	-1.18	5.49E-04	-1.46		
304.1739	1.32	C12H24N4O5					4.80E-05	-1.04
306.0577	5.69	C7 H11 Cl N8 O4	2.61E-03	-1.05	1.10E-02	-1.03		
307.1778	5.31	C17 H25 N O4	5.00E-04	-1.46	9.04E-04	-1.37		
311.1227	6.93	C11H22NO7P	3.35E-04	-1.90	1.76E-03	-1.77		
311.2059	10.49	C17 H29 N O4	1.67E-08	-2.78	4.75E-05	-1.87		
313.1739	6.65	C9 H19 N11 O2	1.00E-08	-2.12	2.01E-05	-1.49		
313.2249	10.49	C13 H27 N7 O2	7.64E-03	-2.09	1.18E-03	-2.60		
325.1895	5.24	C17 H27 N O5			1.36E-03	-2.26		
327.2049	5.49	C18 H25 N5 O			1.44E-03	-1.30		

329.2183	11.68	C21 H31 N S	7.56E-07	-1.77	2.01E-09	-2.36		
334.0875	6.01	C17 H18 O5 S			9.00E-03	-1.48		
335.1328	18.12	C12H21N3O8			1.25E-05	-1.61		
336.1748	5.51	C19 H28 O3 S	2.72E-04	-1.40	3.20E-03	-1.09		
337.2238	4.98	C15 H27 N7 O2			7.65E-03	-1.32		
339.1784	5.52	C23 H21 N3	9.27E-03	-1.16	8.38E-03	-1.12		
339.2385	4.92	C15 H29 N7 O2	7.00E-03	-1.03				
343.2354	5.11	C22 H33 N S			4.76E-04	-1.89	2.89E-04	-1.44
344.0056	3.14	C10 H8 N4 O8 S					1.15E-04	-1.27
345.1171	15.20	C13H19N3O8	2.45E-04	-1.15	3.20E-04	-1.17		
349.0768	1.60	C16H16NO6P					4.85E-03	1.45
353.2189	5.22	C19 H31 N O5			1.55E-03	-1.04		
354.1472	32.91	C14 H30 N2 O2 S3	2.36E-08	-1.00				
355.2352	5.28	C19 H33 N O5	5.02E-06	-1.55	8.15E-05	-1.46		
358.149	20.34	C14H22N4O7			3.29E-03	-1.12		
359.1325	16.90	C14H21N3O8			8.55E-07	-1.36		
364.0983	9.19	C10 H16 N6 O9			3.20E-05	2.17	3.72E-06	2.49
366.142	13.93	C21 H22 N2 O2 S					1.47E-03	1.01
368.1367	5.53	C12 H24 N4 O7 S					1.02E-02	1.14
371.2303	5.41	C19 H33 N O6	2.40E-04	-1.73	7.69E-04	-1.59		
376.1332	4.19	C13H20N4O9	1.01E-02	-1.06	1.43E-03	-1.48		
376.2109	18.54	C19 H28 N4 O4	7.02E-03	-1.23			1.67E-04	1.40
379.0878	2.38	C17 H17 N O9	3.89E-05	1.03	5.66E-06	1.51		
383.1072	4.92	C13H25N3O4S3	2.04E-03	-1.27	4.54E-04	-1.49		
383.1427	10.80	C16H21N3O8	1.79E-04	-1.92	1.85E-03	-1.54		
385.1885	1.24	C23 H31 N S2			1.31E-05	-1.80		
387.1275	1.56	C15 H21 N3 O9	1.36E-03	1.25	7.37E-03	1.08		
389.0759	4.19	C20 H15 N5 S2			4.14E-04	-1.45		
391.9684	8.20	C9 H8 N6 O6 S3			9.91E-04	1.21		
399.1374	17.54	C15H21N5O8	3.84E-05	-1.18	3.46E-05	-1.18		
406.99	10.88	C12 H13 N3 O7 S3	1.41E-04	-1.92			3.90E-08	1.66
410.1659	6.28	C18H26N4O5S1	7.89E-06	-2.88	6.62E-05	-2.37		
412.1271	4.45	C15 H16 N12 O S	2.27E-03	-1.79	1.81E-03	-1.89		
419.1161	5.66	C14 H26 Cl N O11	1.06E-03	-1.25	2.84E-05	-1.76		
422.1715	2.76	C14 H30 N8 O S3	2.21E-03	-1.63	9.79E-03	-1.34		
427.1689	17.92	C14 H25 N11 O S2			3.79E-06	-1.58		
429.1928	29.52	C12 H23 N13 O5			1.88E-04	-1.29		
440.1628	5.56	C17 H28 N8 S3	1.17E-08	-1.71	8.52E-07	-1.84		
448.1164	1.82	C25H20O8			4.53E-04	1.53	3.01E-07	1.65
463.2665	4.71	C25 H41 N3 O S2			7.39E-04	-1.72		
466.0171	3.14	C22 H10 O12	3.90E-03	1.35	9.75E-03	1.38		

468.1955	22.66	C18 H32 N2 O12	3.15E-03	-1.01	9.59E-04	-1.37		
473.1668	1.57	C23H27N3O8	1.93E-04	1.35	1.04E-03	1.26		
473.1943	5.23	C23 H31 N5 O2 S2	1.30E-05	-2.56	8.14E-03	-1.42	2.48E-03	1.14
478.1592	1.98	C22 H26 N2 O10	1.09E-03	1.27				
480.255	8.21	C18 H36 N6 O9			1.07E-03	-1.22		
484.0505	7.68	C16 H20 O15 S	6.14E-09	-2.84	1.31E-03	-1.58	9.14E-03	1.26
484.0565	9.13	C17 H16 N4 O11 S			3.62E-04	1.51	8.45E-10	2.45
494.2503	18.34	C16 H38 N12 S3	6.28E-04	-2.15	1.36E-03	-2.35		
522.1823	6.29	C19 H30 N4 O13	5.58E-06	-2.28			1.92E-04	1.69
523.1551	2.36	C30 H25 N3 O4 S					6.06E-04	-1.05
524.2803	8.95	C28 H44 O7 S	8.30E-06	-1.09	2.70E-04	-1.05		
538.1799	1.48	C25 H34 N2 O7 S2			1.16E-03	-1.01		
540.1785	6.30	C32H28O8	1.27E-06	-2.12	5.89E-05	-1.79		
542.0578	3.09	C23 H10 N8 O9			8.20E-05	-1.56	4.93E-08	-2.00
543.246	1.26	C26 H35 Cl2 N9			3.52E-05	-1.27		
546.1481	2.96	C26 H30 N2 O7 S2	2.10E-07	-2.03			2.94E-02	1.04
558.0037	3.23	C23 H6 N6 O12					0.00E+00	-2.16
559.9756	0.98	C20 H16 O13 S3	1.30E-04	-1.39				
559.9917	35.32	C19 H12 O20			4.72E-06	-1.65	3.88E-04	-1.11
582.1623	5.41	C29 H26 N8 O2 S2			1.75E-04	-1.58		
590.3111	1.31	C33H42N4O6					2.24E-03	-1.52
598.1599	30.02	C19 H34 O21			2.73E-03	-1.10		
604.0146	9.14	C20 H16 N2 O18 S					7.20E-03	1.16
625.7794	31.00	C14 H5 Cl3 N2 O12 S4			8.38E-03	-1.29		
626.1127	4.88	C33 H24 Cl2 N4 O5			7.36E-07	1.38		
643.2733	2.35	C29 H41 N9 O4 S2					3.85E-04	-1.13
654.1917	1.98	C28 H34 N2 O16			8.89E-04	1.60		
655.1921	11.73	C21 H33 N7 O17	3.97E-03	-1.17				
661.298	31.21	C19 H47 N7 O18	5.16E-03	-1.05				
669.5142	6.01	C35 H71 N7 O S2			3.88E-04	2.00	1.18E-02	1.13
672.1861	13.31	C25 H40 N2 O15 S2	1.07E-15	-2.66	2.02E-04	-1.20	1.12E-05	1.46
674.2359	12.84	C29 H42 N2 O14 S	1.68E-03	-1.03				
684.2018	2.40	C22 H36 N8 O15 S			9.08E-05	1.42		
710.0858	3.08	C36 H18 N6 O9 S			4.33E-06	-1.63	3.83E-09	-1.68
713.7605	5.65	C22 H Cl3 N4 O10 S4			6.92E-03	1.10	1.26E-05	1.60
788.2811	18.53	C29 H48 N4 O21	4.16E-03	-1.13	5.67E-03	-1.09		
862.318	21.69	C32 H54 N4 O23	3.09E-04	-1.40	4.05E-05	-1.74		
874.3547	17.06	C34 H58 N4 O22			5.93E-03	-1.15		

Appendix 5. Molecular features in Mexican urine specimens that were statistically significant (based upon pairwise comparisons of molecular features using cut off values of P-value of $\leq$ 0.05 and a FC of $\geq$1) and that differentiated DEN disease states by HILIC LC-MS analysis.

Mass	RT	Calculated formula	DHF/DSS-DF p-value	DHF/DSS-DF FC	DHF/DSS-NEG p-value	DHF/DSS-NEG FC	DF-NEG p-value	DF-NEG FC
151.0295	16.08	C4 H9 N O3 S			2.94E-05	-1.26	1.30E-05	-1.09
169.0934	5.50	C9 H15 N S	9.03E-03	-0.83	3.22E-05	-1.39		
171.089	2.61	C8H13NO3	1.33E-03	1.38			2.15E-03	-2.92
174.1117	25.85	C6H14N4O2	3.68E-05	10.12	3.49E-05	9.57		
175.0037	17.65	C4 H5 N3 O3 S					2.61E-04	-1.19
188.9861	16.10	C3 H8 Cl N O4 S					6.43E-05	-1.04
191.0255	2.23	C6 H9 N O4 S					3.31E-04	-1.21
196.0596	2.57	C7H8N4O3					6.82E-10	-2.42
207.0539	5.15	C10H9NO4			4.70E-04	-1.68	1.71E-04	-1.52
210.0641	2.87	C9H10N2O4					2.62E-04	-1.33
214.0954	5.85	C9 H14 N2 O4			4.01E-07	-2.72		
214.1184	32.25	C7 H14 N6 O2	1.62E-05	1.36			1.87E-02	-2.06
252.0858	3.41	C10H12N4O4			1.00E-07	2.12		
254.1265	17.63	C12H18N2O4			2.15E-04	-1.98	3.99E-04	-1.51
258.1328	24.75	C10 H18 N4 O4	2.41E-04	-0.76	4.45E-07	-1.64		
268.0811	5.37	C10H12N4O5			1.20E-04	1.26		
273.1774	4.48	C14 H27 N O2 S	3.13E-02	-2.56	2.01E-03	-1.45		
284.1745	1.32	C15 H20 N6					2.28E-03	-5.11
287.1519	1.13	C17 H21 N O3	9.23E-02	-0.95	2.13E-04	-2.16		
299.1738	12.70	C16 H21 N5 O					6.99E-05	1.18
303.1233	1.31	C12H21N3O4S1					2.66E-05	-1.16
307.1781	6.76	C17 H25 N O4					3.28E-04	-1.11
315.2387	4.60	C13 H29 N7 O2	2.67E-04	1.53			9.79E-02	-0.48
318.1578	11.54	C17 H22 N2 O4			9.12E-04	1.51		
322.1868	18.69	C17H26N2O4	2.21E-09	1.34			2.01E-02	-2.07
329.1586	4.54	C14H23N3O6	2.01E-03	1.36				
337.0801	1.55	C15 H15 N O8	2.44E-02	-2.50	1.55E-04	-2.47		
353.0381	3.81	C8H15N7O3S3	1.67E-01	-1.43	1.81E-04	-1.21		
368.1359	5.52	C13 H20 N8 O3 S					2.50E-03	1.01
392.2361	0.74	C19 H32 N6 O S	1.19E-03	-1.05	8.68E-05	-1.55	6.76E-02	-0.51
398.3197	1.35	C27H42O2					5.97E-09	-1.32
404.109	1.00	C20 H20 O9					2.37E-04	-1.49
411.275	1.33	C22 H33 N7 O	2.07E-03	1.09			3.55E-04	-0.86
413.293	1.33	C19 H39 N7 O S					9.66E-04	-1.18

415.1583	5.13	C21H25N3O4S1	1.60E-02	-0.67	3.89E-14	-1.36		
421.2062	17.13	C18 H35 N3 O4 S2					1.64E-04	-1.03
437.0892	1.33	C19 H15 N7 O4 S	2.53E-02	0.83	3.05E-04	1.41		
440.9898	8.15	C10 H19 N O10 S4			1.06E-03	-1.05		
456.2956	20.30	C16 H36 N14 S					2.22E-04	1.34
471.9869	11.83	C16 H12 N2 O11 S2	2.88E-02	-0.94	2.75E-04	-1.27		
482.1201	1.20	C25H22O10	4.54E-04	1.16			1.90E-04	-0.85
516.1391	1.50	C25 H20 N6 O7	1.14E-03	5.70	8.62E-05	5.50		
519.3258	10.79	C23 H45 N5 O8	6.90E-02	-1.53	6.20E-03	-2.25		
700.1787	1.83	C29 H36 N2 O16 S	3.94E-02	-1.96	4.59E-03	-2.55		

Appendix 6. Molecular features in Nicaraguan saliva specimens that were statistically significant (based upon pairwise comparisons of molecular features using cut off values of P-value of $\leq$ 0.05 and a FC of $\geq$1) and that differentiated DEN disease states by HILIC LC-MS analysis.

Mass	RT	Calculated formula	DHF/DSS-DF p-value	DHF/ DSS-DF FC	DHF/DSS-NEG p-value	DHF/DSS-NEG FC	DF-NEG p-value	DF-NEG FC
109.053	1.33	C6H7NO			8.15E-03	1.09		
111.0685	1.37	C6 H9 N O	1.67E-02	1.10	5.22E-04	1.39		
111.0798	29.75	C5H9N3					1.44E-03	1.07
115.0458	1.05	C5 H9 N S			7.52E-07	1.90	2.83E-03	1.03
117.0788	12.95	C5H11NO2					5.31E-04	-1.93
118.0781	1.17	C9 H10			1.40E-04	1.16		
126.0317	4.06	C6 H6 O3					1.93E-06	-2.56
131.0943	1.33	C6H13NO2			4.04E-03	-1.04		
143.0583	1.40	C6H9NO3	3.23E-03	1.20				
146.1054	28.46	C6H14N2O2	1.05E-05	1.69			2.13E-06	-1.64
151.0688	26.98	C5 H13 N O2 S					5.61E-03	-1.24
155.0692	27.03	C6H9N3O2	1.46E-03	1.08				
162.0529	7.89	C6H10O5			3.09E-03	-1.98	2.51E-07	-3.00
171.9489	4.16	C5 H Cl N2 O S			3.49E-04	1.87	2.25E-05	1.79
176.044	1.87	C6 H12 N2 S2	1.63E-05	1.27				
178.9895	1.06	C5 H9 N S3			5.66E-04	1.53		
183.0662	22.49	C4 H13 N3 O3 S			8.92E-04	1.03		
186.0634	3.54	C7 H10 N2 O4			7.32E-03	1.02		
190.9546	3.83	C4 H2 Cl N3 O2 S					1.02E-04	1.39
201.1724	1.41	C11H23NO2			7.25E-06	1.33	2.85E-11	2.35
203.1159	16.28	C9H17NO4			4.22E-04	1.37	1.00E-04	1.54
205.1308	2.80	C10H15N5			2.97E-04	1.63		
209.9604	3.84	C6 H7 Cl O2 S2					4.28E-04	1.61
212.0912	27.42	C13H12N2O			5.18E-04	1.15		
215.1167	34.33	C10H17NO4	2.75E-02	1.31	5.91E-05	1.36		
217.1069	29.63	C8H15N3O4					1.61E-04	-1.39
219.1025	2.53	C6 H14 Cl N7	2.32E-05	1.27				
220.1073	1.06	C9 H12 N6 O			3.86E-05	1.21		
227.1888	1.36	C13 H25 N O2					3.30E-04	1.01
228.1336	26.77	C8 H16 N6 O2					7.66E-03	1.14
235.0523	1.02	C9 H17 N S3			9.32E-05	1.17		
242.1018	26.53	C9H14N4O4	5.06E-04	1.30	3.17E-04	1.05		
244.1787	12.86	C12H24N2O3			3.41E-04	1.36	4.64E-11	1.98
250.1776	1.81	C16 H26 S					1.11E-03	-1.03

268.0682	2.45	C12 H16 N2 O S2	5.60E-04	1.47			2.76E-03	-1.00
268.1538	25.95	C12H20N4O3			1.81E-05	1.48		
269.9591	3.66	C7 H7 Cl O7 S			2.39E-03	-1.99		
273.0849	3.78	C11 H15 N O7					6.04E-03	1.02
278.0404	7.23	C8 H11 Cl N4 O5			1.85E-05	-2.76	1.34E-05	-2.01
278.131	33.57	C11H22N2O4S	2.95E-03	1.29	6.05E-05	1.36		
279.9882	4.21	C13 H4 N4 S2	1.73E-03	1.02				
286.0374	4.02	C17 H6 N2 O3					3.33E-03	1.14
291.1383	23.48	C18 H17 N3 O	5.74E-03	-1.82				
293.1742	27.74	C15H23N3O3			2.71E-04	1.19		
294.004	4.17	C9 H10 O9 S					5.83E-03	-1.56
296.1836	4.33	C14 H32 O2 S2					1.79E-03	1.55
300.15	30.23	C7 H24 N8 O S2	9.26E-05	1.18			1.35E-05	-1.80
300.2094	1.08	C20H28O2					3.65E-03	-1.01
301.2001	13.86	C14H27N3O4	2.06E-03	-1.10	5.21E-04	-1.36		
307.2874	1.26	C20 H37 N O					7.32E-04	1.24
309.3031	1.23	C20 H39 N O			6.79E-03	1.28	1.14E-02	1.03
311.318	1.25	C20 H41 N O			4.33E-04	1.03	1.01E-04	1.10
315.2172	13.81	C15H29N3O4					8.11E-03	-1.22
317.1939	14.03	C14H27N3O5					1.01E-03	-1.18
320.1236	5.04	C21 H20 O S			4.40E-04	1.82		
320.1549	41.06	C9 H20 N8 O5			6.31E-03	1.29		
322.1448	23.51	C15 H30 O S3					3.62E-03	1.25
324.2779	1.38	C19 H36 N2 O2			2.09E-10	2.34	2.76E-16	2.67
325.3709	11.99	C22 H47 N	2.10E-03	1.25	1.25E-03	1.04		
335.3183	1.23	C22 H41 N O			2.07E-03	1.62	2.10E-03	1.47
337.1793	41.32	C12 H27 N5 O4 S			7.39E-03	1.09		
337.3348	1.23	C22 H43 N O			1.31E-03	1.38		
342.2862	12.97	C15 H34 N8 O			2.73E-03	1.79		
347.193	32.53	C12 H25 N7 O5	1.43E-05	1.05			1.36E-06	-0.91
357.1069	2.78	C16 H15 N5 O5					6.22E-03	1.01
358.3083	1.12	C21H42O4					3.95E-03	-2.28
359.2043	12.89	C16H29N3O6	1.49E-04	-1.04			4.01E-05	0.85
360.1913	32.14	C15H28N4O6					1.16E-02	-1.15
363.1871	41.64	C13 H29 N7 O S2	3.86E-04	-1.44			4.37E-06	1.50
364.2233	2.05	C25 H32 S	4.36E-07	1.59			3.42E-05	-0.95
365.1322	11.63	C15 H19 N5 O6	2.48E-03	-1.38				
369.3244	1.22	C22 H43 N O3			7.51E-04	2.25	1.33E-03	2.07
371.3388	1.36	C18 H41 N7 O					7.53E-03	1.52
376.0509	1.39	C12 H8 N8 O7					2.25E-04	-1.23
380.0721	7.75	C14 H16 N6 O3 S2					1.33E-03	-1.03

384.1966	26.98	C13 H32 N6 O3 S2	1.90E-05	-1.32			5.01E-04	1.04
385.3188	1.27	C18 H39 N7 O2			5.38E-03	1.72	4.59E-03	1.75
389.2112	19.38	C17 H27 N9 S					4.36E-03	1.26
396.1948	1.15	C25 H24 N4 O			1.20E-05	1.19		
399.2026	2.20	C16 H37 N3 O2 S3			2.10E-04	2.06		
403.1994	1.18	C22 H29 N O6					7.91E-04	1.12
406.1852	17.67	C19H26N4O6			3.50E-03	1.46		
409.3191	2.97	C24 H43 N O4	4.91E-05	1.43	3.10E-05	1.20		
414.2749	2.86	C22 H42 N2 O S2	2.19E-04	-1.32				
415.1092	1.85	C17 H21 N O11	2.21E-03	1.74				
422.2169	40.08	C20H30N4O6			3.57E-03	-1.14		
426.382	12.45	C21 H46 N8 O			1.46E-03	1.08		
428.2295	41.42	C13 H28 N14 O S	5.76E-03	1.37	2.45E-04	1.31		
444.1787	1.22	C26 H20 N8	1.94E-03	1.16	2.91E-03	1.19		
446.1993	42.83	C15 H38 N6 O S4			9.25E-05	-1.22		
450.3545	1.08	C23 H51 Cl N4 S	9.59E-04	1.23			4.87E-03	-1.12
460.0779	4.16	C22 H24 N2 O S4			4.72E-03	-1.06	2.92E-06	-1.37
463.2432	23.35	C26 H33 N5 O S	3.99E-03	1.54			5.23E-03	-1.43
466.243	33.87	C15 H34 N10 O5 S	5.77E-03	1.50				
471.1963	5.89	C19 H29 N5 O9	3.38E-03	-1.46	6.81E-03	-1.17		
471.2072	22.23	C15 H29 N13 O S2	2.46E-03	1.42				
487.9527	3.39	C16 H8 O16 S			3.68E-04	1.23		
491.1803	1.27	C26 H29 N5 O S2	1.19E-05	1.25	3.35E-06	1.43		
496.2543	39.89	C16 H36 N10 O6 S	3.95E-04	2.45	7.90E-03	1.25	2.93E-02	-1.20
500.0913	2.01	C24 H25 Cl N4 S3			6.68E-04	1.48	9.14E-09	1.52
505.4815	1.26	C29 H59 N7			8.27E-04	-1.12		
506.2486	32.52	C22 H38 N2 O11					2.96E-03	-1.74
509.2182	26.41	C16 H31 N9 O10	2.33E-03	1.22				
511.3018	1.21	C24 H42 Cl N7 O3			1.62E-03	-1.02	1.38E-05	-1.08
512.0229	4.16	C15 H16 N2 O16 S			2.64E-04	1.64	3.89E-06	1.76
515.2795	40.59	C16 H33 N15 O5			5.43E-05	-2.75		
527.2396	26.67	C15 H33 N11 O10	4.80E-05	1.82	6.34E-05	2.00		
531.2834	1.15	C30 H45 N O3 S2			3.84E-03	1.04		
532.2299	41.43	C24 H40 N2 O7 S2					2.84E-04	1.06
532.2407	28.23	C27 H36 N2 O9	2.00E-03	1.01	1.31E-04	1.15		
534.2796	24.40	C36 H39 Cl N2					5.90E-03	-1.22
564.2226	1.23	C27 H40 N4 O3 S3	3.28E-04	1.28	7.31E-03	1.11		
567.1577	12.81	C17 H33 N3 O16 S			2.72E-07	-1.85	7.58E-05	-1.55
572.1831	2.00	C29 H28 N6 O5 S	2.14E-04	-1.81				
575.3072	34.72	C39 H37 N5			1.03E-03	1.15		
578.4942	1.35	C33 H62 N4 O4					7.30E-03	1.30

580.269	33.46	C22 H40 N6 O12	6.61E-03	1.13				
589.7871	32.68	C22 H Cl3 N2 O4 S4					2.70E-05	-1.26
591.2627	33.45	C23 H29 N17 O3			2.46E-03	1.15	4.08E-04	1.01
613.8054	40.64	C19 H2 O14 S5	1.58E-03	1.35				
619.2977	27.79	C25 H33 N17 O3	1.16E-03	1.87				
633.4456	1.53	C31 H63 N5 O6 S	2.23E-05	1.44	1.05E-03	1.57		
642.1577	6.53	C29 H19 Cl N16 O	2.74E-04	1.91	6.04E-04	1.89		
646.4519	1.15	C37 H58 N8 S	1.21E-04	1.39				
657.8577	34.78	C19 H2 N2 O21 S2					4.42E-04	1.67
658.8388	27.45	C27 H N O12 S4	1.62E-02	2.35				
663.4557	2.27	C39 H69 N O S3			1.25E-05	-1.60	5.97E-04	-1.17
668.2805	7.35	C32 H48 N2 O9 S2					3.34E-03	-1.09
701.3051	12.74	C35 H59 N O3 S5	5.04E-03	-1.38			1.63E-03	1.22
705.2826	18.01	C28 H47 N7 O10 S2					5.79E-03	1.04
707.2489	14.95	C36 H41 N3 O10 S			6.36E-03	1.30		
710.3369	27.71	C35 H58 N4 O3 S4	4.78E-04	2.48				
711.2862	8.47	C34 H45 N7 O6 S2	2.87E-03	1.25				
712.3566	27.44	C34 H48 N8 O9	1.04E-03	1.14			2.04E-08	-1.42
715.5593	1.36	C41 H78 Cl N O6			2.03E-04	-1.03		
726.4532	2.43	C37 H70 N6 S4	2.64E-08	-1.58	2.15E-04	-1.41		
742.868	35.46	C31 H8 Cl3 N7 O2 S4			3.59E-05	-1.22	2.07E-07	-1.32
743.3567	21.31	C37 H53 N5 O9 S			2.38E-03	1.07		
750.3661	25.43	C41 H58 N4 O3 S3	1.56E-04	-1.69				
754.3418	40.80	C47 H50 N2 O5 S			2.20E-05	1.24		
767.3573	27.85	C36 H65 N O8 S4			8.71E-05	1.62	1.27E-04	1.36
775.8925	41.98	C41 H9 Cl O7 S4	1.36E-05	1.87			8.87E-07	-1.72
779.5447	13.50	C47 H77 N3 O2 S2					1.44E-03	-1.35
811.4324	1.59	C46 H69 N O5 S3					4.81E-07	-1.11
883.4372	38.25	C48 H61 N5 O11					1.23E-04	-2.01

Appendix 7. Molecular features in Mexican saliva specimens that were statistically significant (based upon pairwise comparisons of molecular features using cut off values of P-value of $\leq$ 0.05 and a FC of $\geq$1 and that differentiated DEN disease states by HILIC LC-MS analysis.

Mass	RT	Calculated formula	DHF/DSS-DF p-value	DHF/DSS-DF FC	DHF/DSS-NEG p-value	DHF/DSS-NEG FC	DF-NEG p-value	DF-NEG FC
105.0429	2.13	C3H7NO3			8.15E-04	4.31		
108.094	1.33	C8 H12			6.61E-03	2.92		
109.0282	2.97	C4 H3 N3 O			3.13E-04	1.25	7.83E-03	2.60
109.0642	27.75	C5 H7 N3			5.74E-03	3.18		
110.0733	1.29	C7H10O			4.83E-03	3.18	1.34E-02	2.97
111.0686	1.45	C6 H9 N O					7.54E-03	3.02
113.0842	7.37	C6H11NO			6.81E-03	3.91		
120.0436	1.38	C5H4N4			2.13E-03	4.51	3.21E-03	2.81
123.032	6.91	C6H5NO2			1.62E-03	1.46	1.96E-03	3.28
125.0147	8.18	C2H7NO3S					8.86E-03	2.86
126.0428	1.91	C5H6N2O2					7.16E-03	2.91
129.0426	3.01	C5H7NO3					1.40E-02	2.83
129.0578	1.21	C9H7N			7.98E-04	3.73	8.38E-05	4.94
130.1104	20.38	C6H14N2O			1.63E-03	3.89	5.30E-03	1.52
136.0386	7.15	C4H8O5	2.04E-03	1.28	9.45E-04	1.50		
143.1309	1.47	C8 H17 N O					1.36E-02	2.67
145.053	1.29	C9H7NO			3.52E-03	3.14	3.02E-04	4.05
151.0495	10.67	C5H5N5O			4.83E-03	2.99	2.41E-03	3.19
152.0334	2.98	C5H4N4O2			3.64E-04	4.25	8.14E-04	3.97
155.0695	27.85	C6H9N3O2			3.73E-03	3.74		
155.0945	1.45	C8H13NO2			2.47E-03	1.32	5.53E-04	4.26
157.1094	3.31	C8H15NO2			4.95E-07	2.48	7.13E-03	2.85
158.0439	2.48	C4H6N4O3			2.43E-03	4.11		
158.0692	18.83	C6 H10 N2 O3			1.16E-02	2.67	3.31E-03	3.51
161.0685	1.94	C6H11NO4			1.47E-03	3.71	4.85E-03	3.41
163.0839	3.09	C6 H13 N O4			1.44E-04	4.48	3.21E-04	4.47
171.0895	1.42	C8H13NO3			1.60E-03	3.89	8.32E-03	3.00
172.0848	19.66	C12H12O					1.06E-02	3.35
172.1212	7.74	C8 H16 N2 O2			1.03E-04	3.32	2.57E-04	4.07
173.1044	1.42	C8H15NO3			2.82E-03	3.32	1.27E-03	4.29
175.0632	1.16	C10H9NO2			1.19E-04	4.58	3.54E-04	2.84
175.0957	18.76	C6H13N3O3			1.32E-02	2.69	4.35E-03	3.27
178.1356	1.14	C12H18O			2.78E-03	3.02	4.65E-03	3.04
184.121	8.47	C9 H16 N2 O2			1.85E-02	2.28	6.34E-03	2.52

185.0688	6.26	C8 H11 N O4					7.99E-03	3.00
186.0505	3.07	C4 H6 N6 O3			5.19E-03	1.72	7.49E-03	3.70
187.1206	1.42	C9H17NO3			6.10E-03	2.23	4.15E-03	3.36
188.9642	4.51	C6 H4 Cl N O2 S			1.35E-07	1.63	7.14E-04	2.41
190.07	5.71	C5 H10 N4 O4			6.43E-03	2.96	4.99E-03	2.97
198.1369	4.49	C10 H18 N2 O2			1.59E-04	4.18	1.34E-03	2.16
199.1206	1.41	C10H17NO3			9.24E-03	3.36	9.10E-03	2.82
202.0455	4.45	C4 H6 N6 O4			1.60E-02	2.75	1.01E-02	2.86
202.1429	28.70	C8H18N4O2			9.06E-03	3.09		
211.0587	1.70	C8H10ClN5			3.15E-04	5.64	5.64E-04	5.08
217.1064	6.04	C8H15N3O4			1.29E-02	3.26		
218.0194	4.56	C7 H10 N2 O2 S2			3.67E-04	3.40	1.94E-03	3.98
219.1105	1.88	C9H17NO5			5.24E-05	4.91	3.04E-04	4.25
220.2186	1.28	C16 H28	6.63E-05	-1.03				
229.1791	26.90	C11 H23 N3 O2			8.89E-04	3.44	2.57E-03	1.42
241.93	2.99	C5 H3 Cl O7 S	5.31E-03	4.17	5.25E-03	4.75		
242.1265	2.46	C16H18O2			9.41E-03	3.88	1.50E-03	2.68
244.0697	2.98	C9H12N2O6			2.08E-03	3.74	5.27E-04	4.32
250.0575	2.31	C9H15O6P			4.79E-03	3.39	1.40E-03	4.08
250.1917	1.13	C9H17NO3			1.68E-02	2.31		
251.008	18.40	C4 H9 N7 S3					8.08E-03	3.21
251.9211	2.86	C11 H2 Cl2 O S			4.33E-03	2.50	1.50E-02	1.79
252.0861	6.48	C16H12O3			1.19E-03	4.33	4.91E-04	4.41
253.9643	3.01	C7 H7 Cl O6 S	1.54E-03	4.64	4.39E-03	4.71		
256.2399	1.20	C16H32O2			4.81E-03	4.71	4.78E-04	4.81
260.1367	2.28	C11H20N2O5			5.92E-06	3.62	7.16E-04	4.38
265.0481	9.11	C6 H11 N5 O5 S					6.26E-03	3.66
268.0805	5.72	C10H12N4O5			9.22E-04	4.19	2.82E-04	4.09
269.1377	20.78	C12H19N3O4			1.73E-03	-2.00		
275.1006	2.49	C11 H17 N O7			2.27E-03	3.33	1.28E-03	3.51
276.209	1.14	C18H28O2			4.85E-03	2.49	8.85E-03	2.81
278.2241	1.15	C18H30O2			3.10E-03	2.76	5.02E-03	3.20
280.2406	1.12	C18H32O2					9.32E-03	3.04
282.9557	2.99	C7 H6 Cl N O7 S	6.61E-03	3.99	6.39E-03	4.62		
283.117	6.80	C12 H17 N3 O5	1.63E-04	-2.17			1.72E-04	1.61
288.0329	4.44	C13H14Cl2O3			1.20E-04	4.56	5.36E-03	1.96
293.111	3.07	C11H19NO8			6.37E-06	4.27	2.28E-05	5.17
294.2198	1.15	C18H30O3			7.63E-04	3.52	4.77E-03	2.97
296.2349	1.14	C18H32O3			5.54E-03	3.12	1.09E-02	2.31
298.2508	1.13	C18H34O3			4.31E-03	1.07	7.99E-04	3.41
300.2081	1.20	C20H28O2					7.49E-03	2.94

302.2252	1.16	C20H30O2					1.12E-02	2.72
304.2391	1.14	C20H32O2			2.03E-02	2.09	1.37E-02	2.62
306.0369	5.74	C11 H10 N6 O S2			2.17E-04	4.79	5.88E-04	4.00
308.2192	2.27	C15 H32 O6			1.18E-03	1.46	1.13E-02	2.20
316.2036	1.16	C20H28O3			1.33E-03	3.99	3.85E-03	3.02
320.2349	1.17	C20H32O3			3.79E-03	1.35	5.36E-03	2.79
331.0671	3.13	C10H14N5O6P			3.04E-20	3.55	1.10E-05	5.26
336.2296	1.17	C20H32O4			8.18E-05	1.60	2.28E-03	2.64
354.0617	1.12	C17 H22 S4			8.04E-04	3.72	9.61E-03	2.86
364.2816	1.47	C23 H40 O S			4.28E-04	4.68	6.03E-03	2.01
365.1321	11.10	C14 H23 N O10					6.62E-03	2.63
372.2861	1.30	C22 H44 S2	3.86E-07	-1.09				
375.0438	8.19	C11 H17 N7 S4					7.36E-03	2.98
376.1381	4.62	C17H20N4O6			4.53E-03	3.02	2.81E-03	2.77
382.1604	26.89	C15 H22 N6 O6	3.43E-03	2.27	6.02E-10	3.27		
383.1426	10.96	C16H21N3O8			5.56E-03	2.71	6.91E-04	3.26
386.174	1.23	C15H26N6O4S1			1.95E-08	1.21	4.25E-03	2.69
402.3344	1.33	C27 H46 S	1.46E-07	-1.88	2.94E-07	-1.48		
405.9971	3.01	C16 H11 Cl N4 O3 S2	6.08E-03	4.01	6.01E-03	4.63		
414.2455	8.99	C15 H30 N10 O4					7.13E-03	3.91
417.2217	7.35	C17 H31 N5 O7			7.26E-03	2.95		
418.0329	3.03	C18 H7 Cl N8 O3	9.73E-04	4.84	1.89E-03	5.28		
420.1643	5.92	C25H24O6					1.42E-02	3.26
421.0991	10.91	C15 H23 N3 O7 S2			1.28E-02	1.26	4.49E-03	3.18
426.2951	1.47	C20H38N6O4			5.26E-03	3.81		
430.0654	1.17	C21 H22 N2 S4			3.57E-06	1.55	1.03E-02	2.03
433.1818	25.76	C17 H31 N5 O4 S2			1.10E-04	5.07	3.09E-06	2.49
444.1115	1.10	C21 H32 S5			3.07E-03	3.14		
447.1039	1.14	C35 H13 N			6.54E-06	1.47	1.06E-02	2.69
460.9209	3.41	C17 H5 Cl2 N5 O3 S2			1.11E-02	4.59		
475.2576	1.28	C20 H33 N11 O S			3.10E-04	3.13	9.95E-03	2.55
500.0585	8.18	C13 H24 N8 O3 S5					1.26E-02	2.72
504.0796	1.16	C30H16O8			9.21E-06	1.53	6.19E-03	2.73
515.179	12.63	C27 H33 N O5 S2	7.65E-04	5.09	1.97E-03	5.26		
518.1287	1.13	C33 H23 Cl O4			1.28E-03	3.01	7.86E-03	2.66
522.1066	1.16	C26 H18 N8 O S2			2.30E-03	1.19	1.35E-02	2.48
536.5276	1.44	C34 H68 N2 O2	3.75E-03	-1.03				
545.1953	13.56	C17 H27 N11 O10			6.99E-03	1.42	3.11E-03	2.43
578.0981	1.15	C31 H19 Cl N4 O6			1.32E-06	1.63	5.96E-03	2.92
583.1516	13.38	C17 H33 N3 O17 S			4.27E-03	3.95	2.65E-04	4.30
592.1469	1.14	C39 H26 Cl2 N2			1.01E-03	1.01	1.25E-02	2.54

625.0728	8.18	C18 H31 N3 O13 S4					1.06E-02	2.89
627.186	1.14	C37 H30 Cl N5 O S			5.27E-04	1.37	1.84E-04	4.63
630.1153	2.91	C20 H22 N8 O16			1.84E-11	3.74	8.46E-06	5.85
636.2999	1.08	C29 H48 O15			3.13E-05	-3.91	3.85E-03	-2.32
698.4025	2.61	C37 H58 N6 O3 S2	4.98E-03	-1.59	2.09E-07	-2.23		
701.2079	1.12	C44 H32 Cl N3 O4					6.81E-03	2.82
701.4929	1.41	C32 H67 N11 O2 S2			3.64E-04	4.11	8.30E-04	3.39
718.4221	1.11	C40 H66 N2 O3 S3	9.29E-05	-1.16	2.99E-05	-1.46		
725.5234	1.11	C36 H75 N3 O9 S	4.08E-11	-1.85	6.07E-12	-1.33		
726.1318	1.12	C48 H23 Cl N2 O4			2.14E-03	3.26	8.98E-03	2.70
728.2822	1.07	C39 H32 N14 O2	1.08E-08	-1.11				
741.4284	18.14	C35 H55 N11 O7	2.29E-03	1.36				
745.5178	1.41	C34 H71 N11 O3 S2			1.76E-03	3.80	8.32E-04	3.90
775.2239	1.11	C50 H34 Cl N3 O4			1.56E-02	2.71	9.85E-03	2.59
921.0022	1.11	C28 H27 N O30 S2			3.46E-03	3.41	3.74E-03	3.21