



**UNIVERSITY
OF MALAYA**

The Leader in Research & Innovation

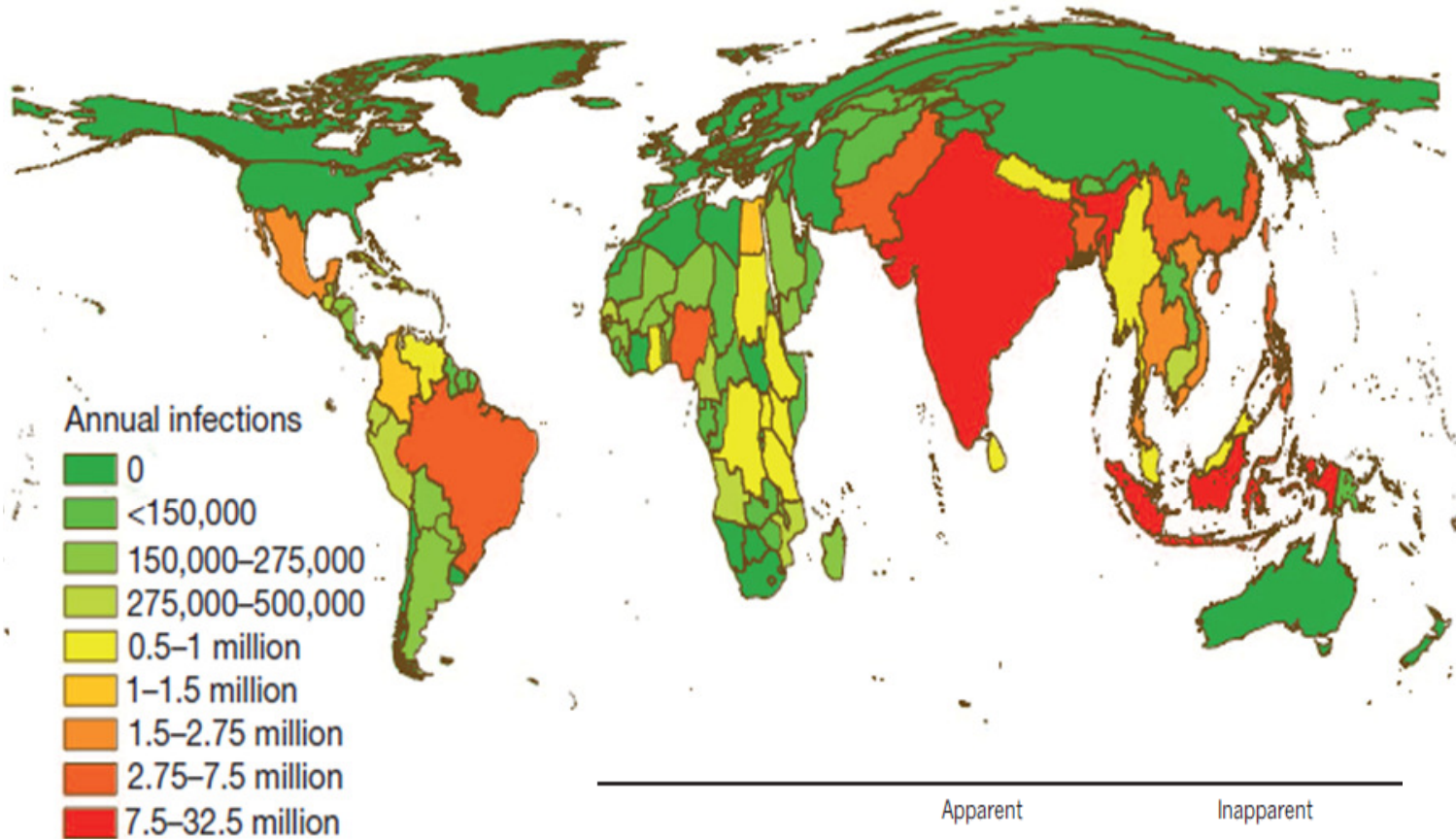
Dengue infection : Cytokine profiles and modulation of the microvascular endothelium

Shamala Devi Sekaran

**3rd International Conference & Exhibition on Clinical and
Cellular Immunology**

Baltimore Sept 29th – Oct 1st 2014

Dengue: Disease Burden (World)



390 million cases /year

Apparent: 96 million

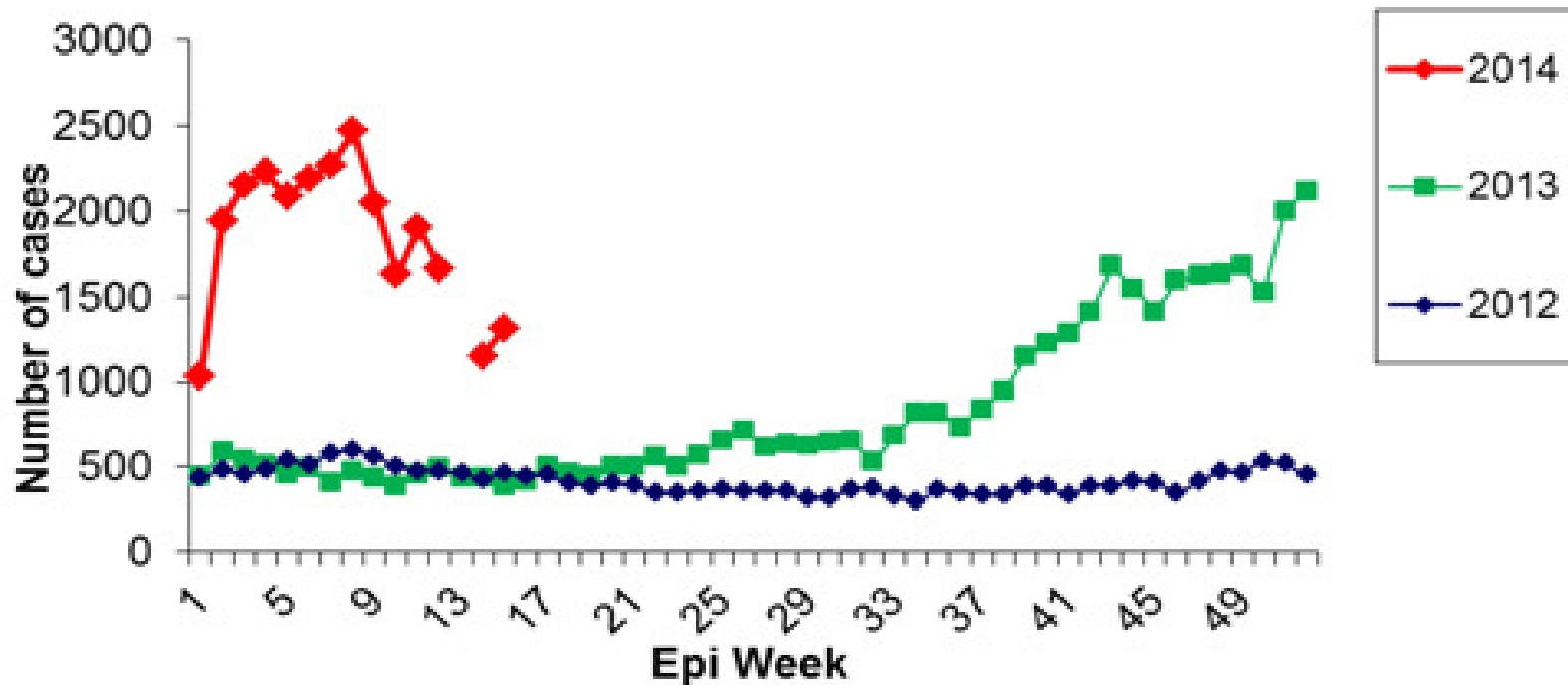
Inapparent: 294 million

	Apparent	Inapparent
	Millions (credible interval)	Millions (credible interval)
Africa	15.7 (10.5–22.5)	48.4 (34.3–65.2)
Asia	66.8 (47.0–94.4)	204.4 (151.8–273.0)
Americas	13.3 (9.5–18.5)	40.5 (30.5–53.3)
Oceania	0.18 (0.11–0.28)	0.55 (0.35–0.82)
Global	96 (67.1–135.6)	293.9 (217.0–392.3)

Bhatt et al., 2013

Dengue: Disease Burden (Malaysia)

Dengue in Malaysia, 2012-2014



http://www.wpro.who.int/emerging_diseases/dengue.epicurves/en/index.html

(Source: National Center for Laboratory and Epidemiology, Ministry of Health)

Malaysia up to 13 April 2014 (Source: Ministry of Health)

Aug 2014

-65672 cases with 128 deaths

Aug 2013

-18099 cases with 35 deaths

http://www.moh.gov.my/pr_categories/1/press_releases

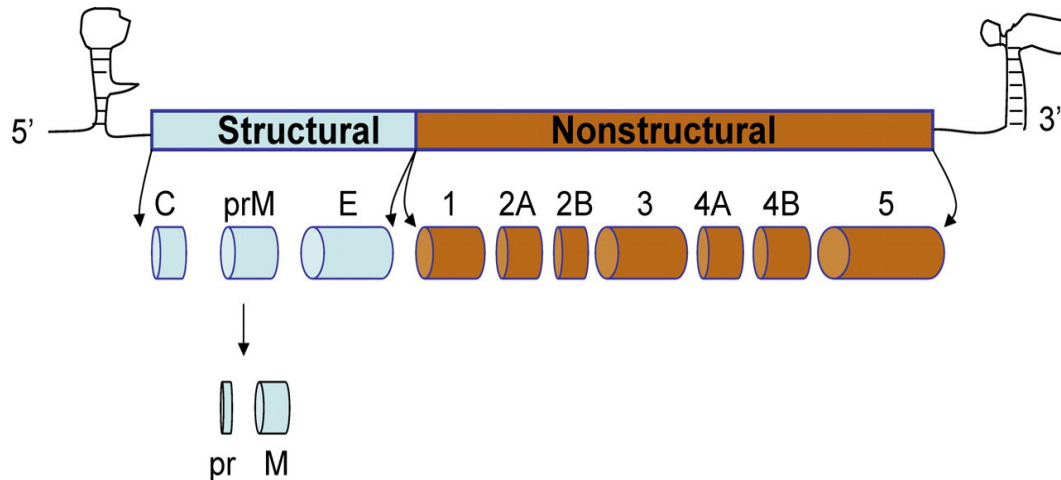
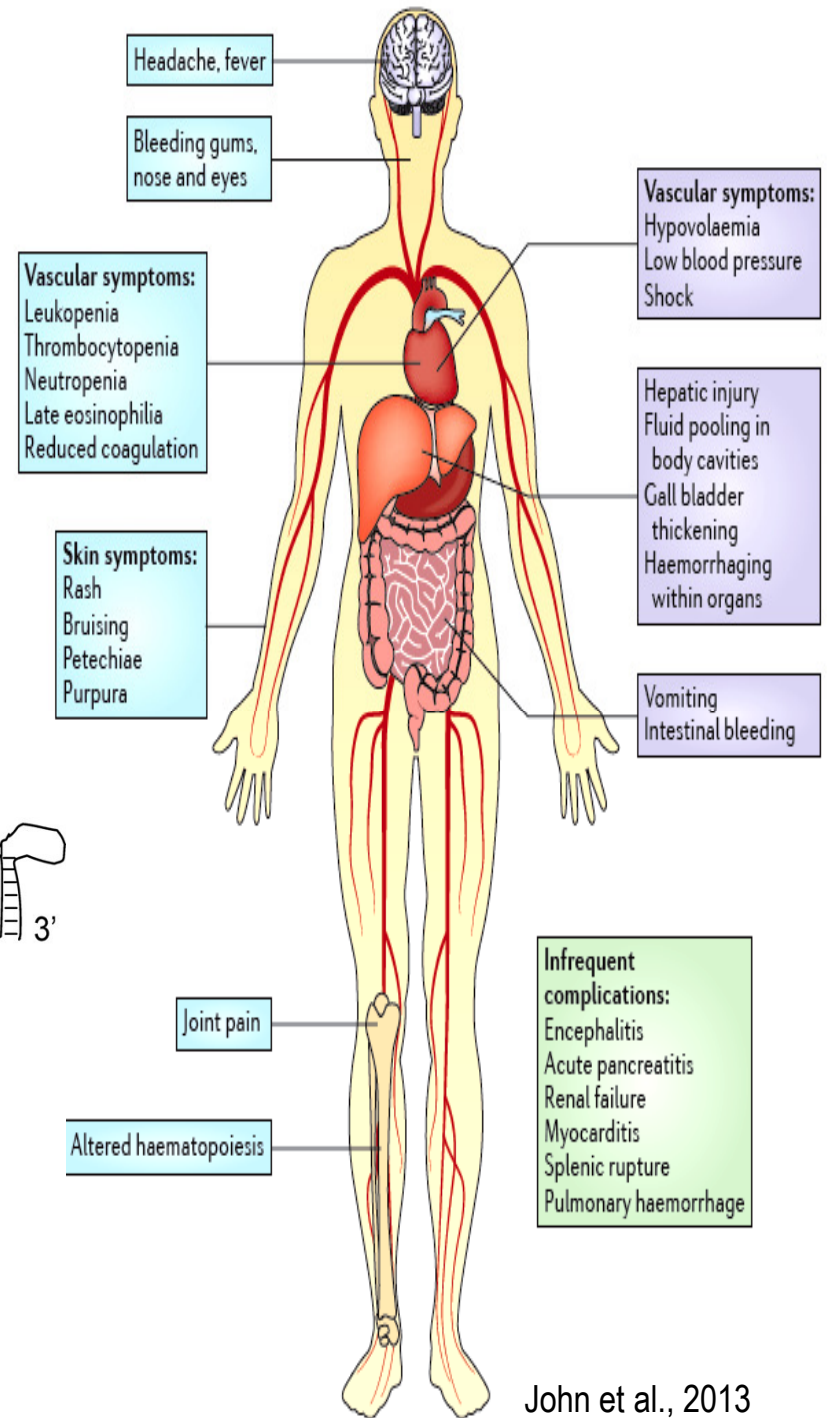
Dengue Virus → Transmitted by

- + ssRNA virus,
- Flaviviridae
- 4 serotypes
- Genome:
 - 3 structural
 - 7 non-structural

Aedes aegypti



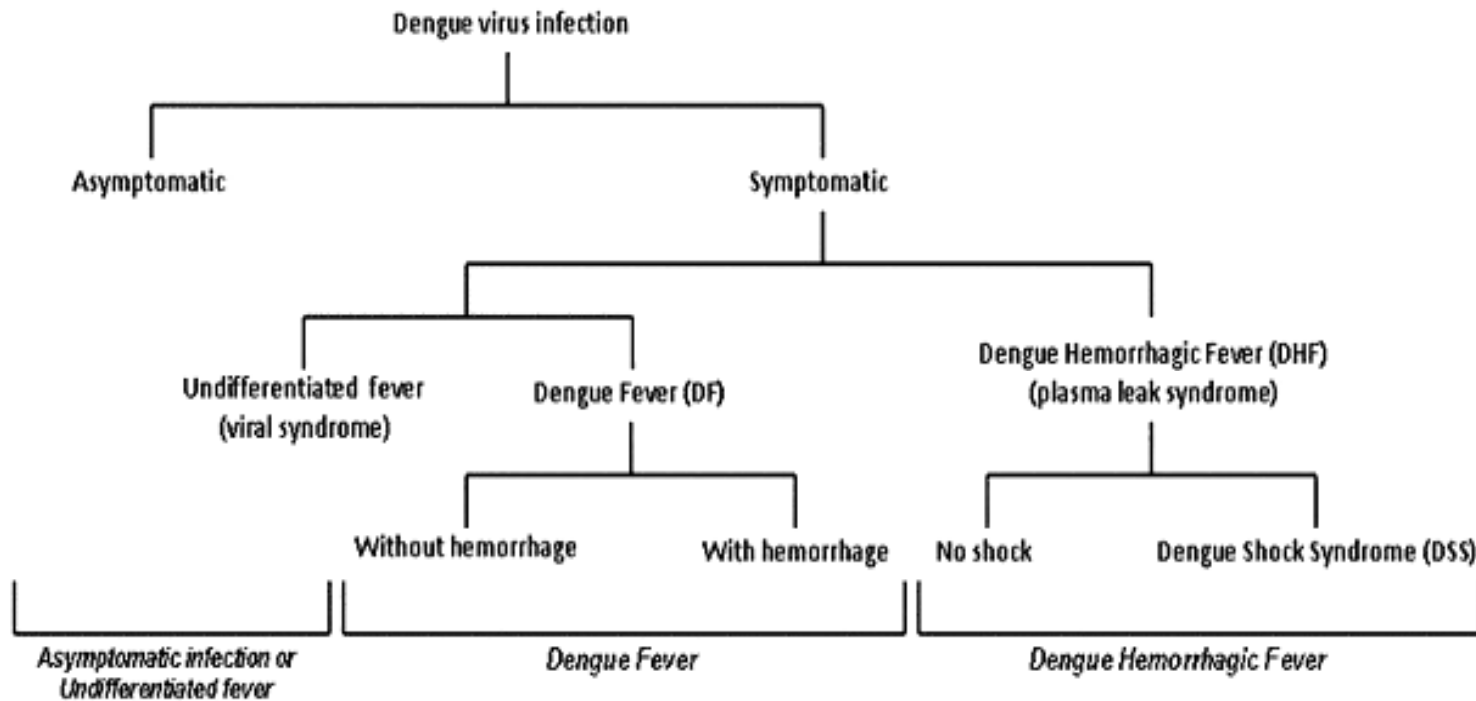
Aedes albopictus



Noisakran and Perng 2008

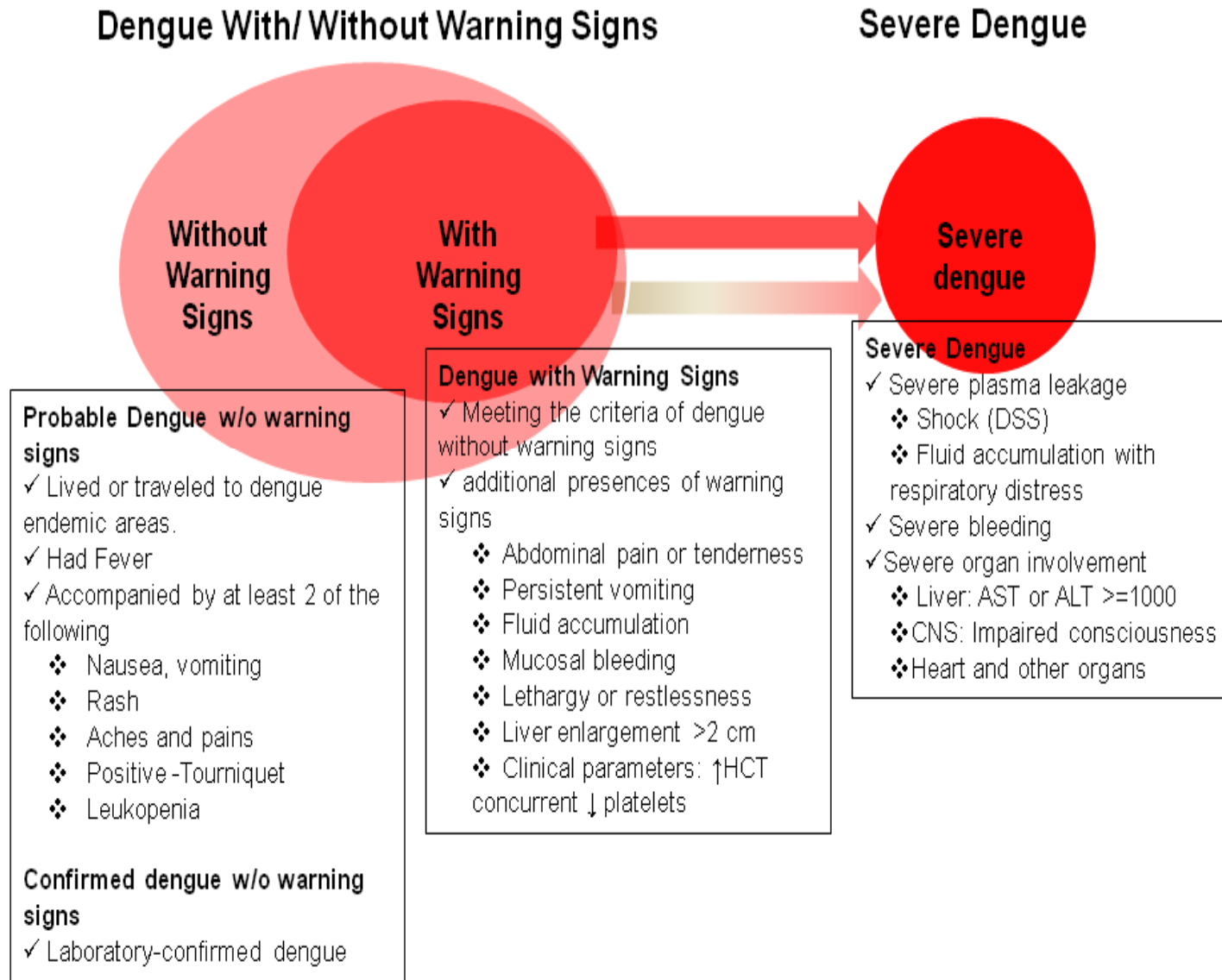
John et al., 2013

Dengue Classification (WHO 1997)



*Adapted from *Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control*. 2nd edition. WHO, Geneva, 1997

Revised Dengue Classification (WHO 2009)



Study: Classification and demography

Ethical clearance

UM (782.90), HA (NMRR-10-683-6420), HTAR (NMRR-10-683-6420) and USF(Pro00000425)

Dengue suspected patients @ HA and HTAR (606)

Criteria: (i) ≥ 14 years with consent
(ii) clinical symptoms suggestive of dengue (WHO 2009)

Refusal : 94

Study entrants: 512

Withdrew: 8

Classification : (i) clinical diagnosis by WHO 2009 dengue classification
(504) (ii) hospital diagnostic assays : IgM and NS1

ND: 47

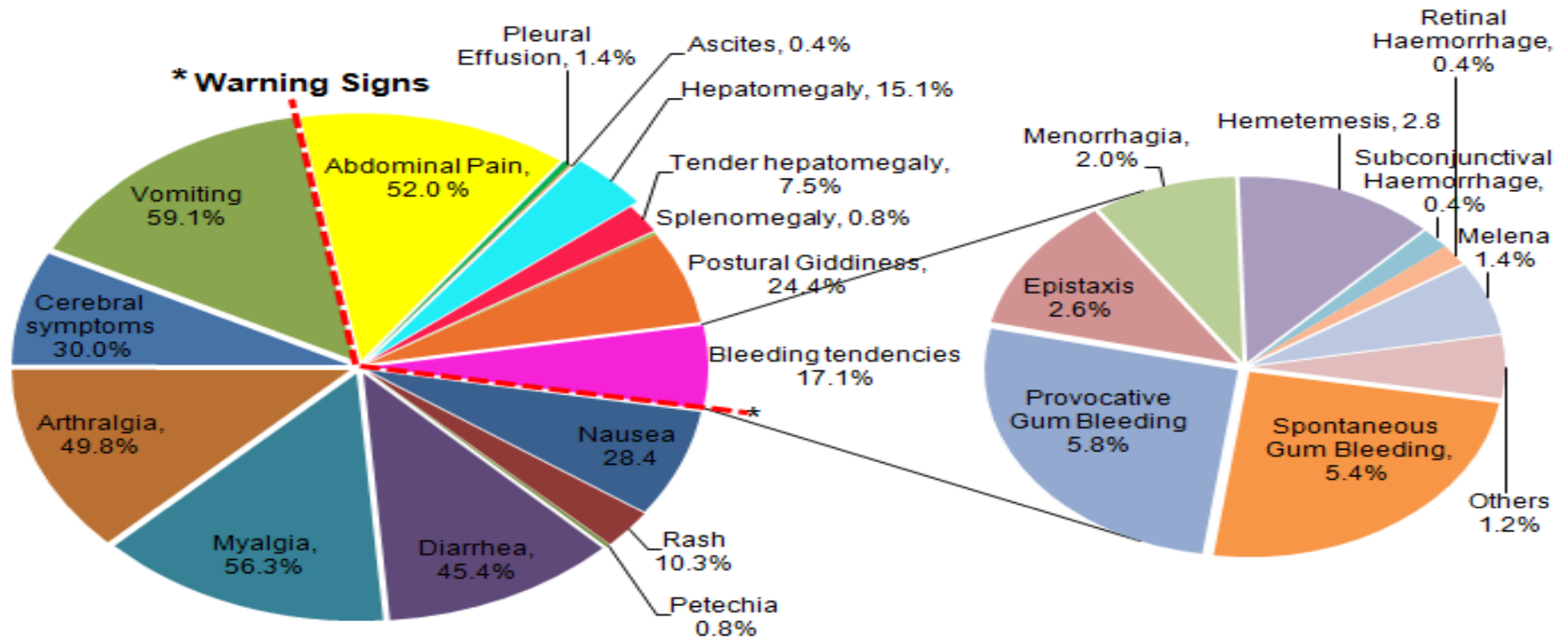
DwoWS: 64

DwWS: 388

SD: 5

	ND	DwoWS	DwWS	SD
No. Patients	47	64	388	5
Age (Mean \pm SEM)	15-81 (29.1 \pm 2.0)	13- 60 (29.9 \pm 1.5)	12 - 77 (29.5 \pm 0.6)	22 - 39 (27.5 \pm 2.6)
Sex				
Female	13	11	98	1
Male	34	53	290	4
Race				
Malay	31	37	259	3
Chinese	3	11	52	2
Indian	8	7	44	0
Others	5	9	33	0
Pregnancy	1	0	2	1
Previous DENV Infection^b	1	2	22	0

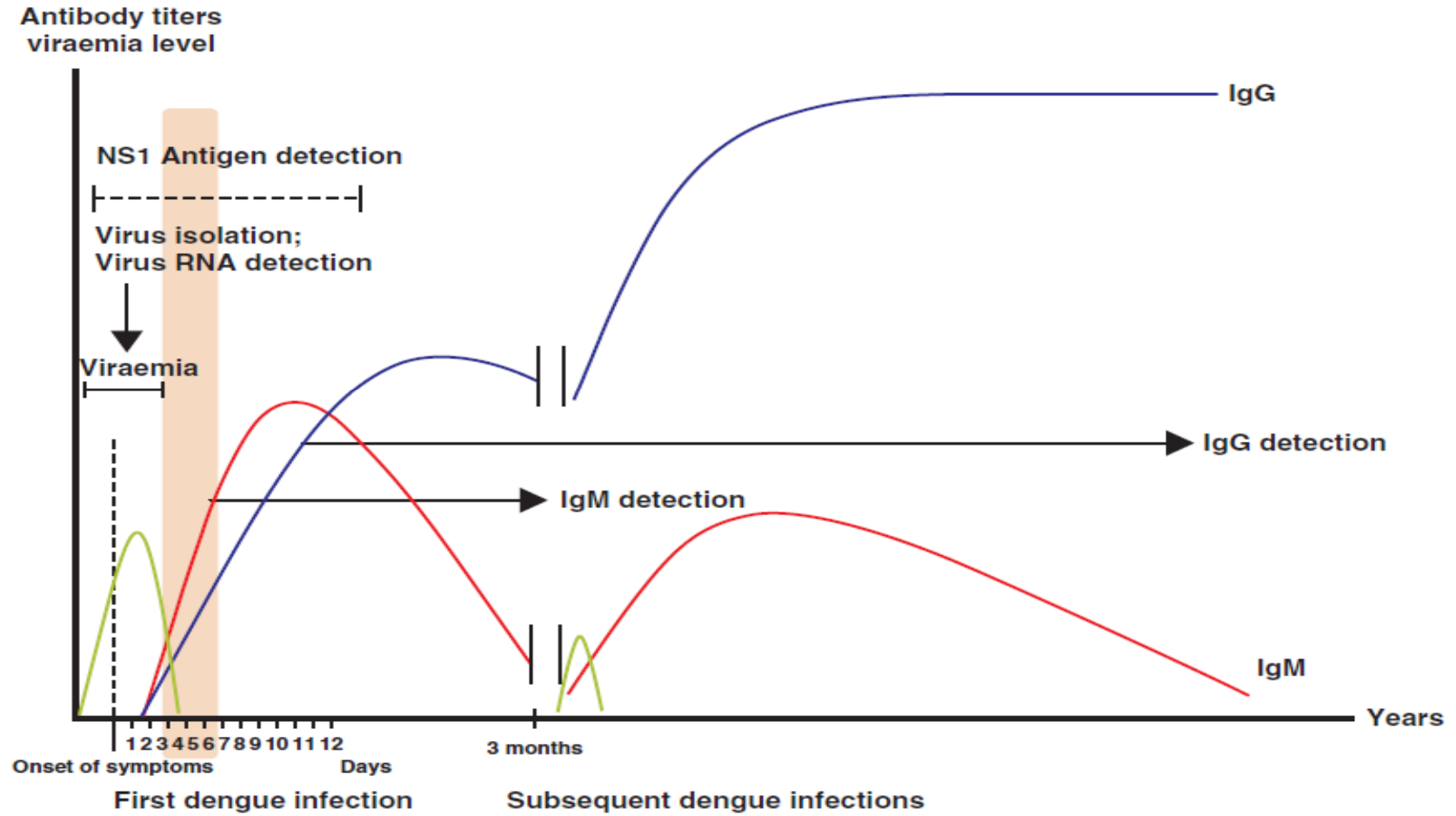
Study: Clinical Symptoms



	ND	DwoWS	DwWS	SD
No. Patients	47	64	388	5
Diagnosed with hepatitis	3	16	165	2

37%

Diagnosing Dengue



Study: Laboratory Diagnostics

Hospital Diagnostics

	Negative	Positive	Undetermined	
IgM	155	225	124	44.4%
NS1	80	52	372	26.0%

Dengue Confirmation algorithm

CONFIRMED DENGUE	PRESUMPTIVE DENGUE
1) DENV RNA and/or Ag	1) Only IgM
2) IgM seroconversion	2) HI >1280 (single sample)
3) 4X rise in HI titers	
4) Combination of above	

Lab diagnostics	TOTAL	ND	DwoWS	DwWS	SD
Positive	311	12	37	259	3
Presumptive	54	5	6	43	0
Negative	133	29	21	81	2

37%
misdiagnosed
as ND

1°- 221 patients
2°- 140 patients
N/A- 5 patients

RNA +ve: 49 patients
Serotypes: DENV1 (69.4%)
DENV3 (30.6%)

NS1: 217 patients

IgM seroconversion: 49 patients

Summary

- Demographic – in accordance to national profiles
- ↑ in **patients with WARNING SIGNS**
- Clinical + lab diagnosis = **A MUST**
- ↑ silent infection- only 5% aware but 27.8% had 2° infections
- Clinical parameters – no differentiating ability between without and with warning signs except of TP and TB
- Thrombocytopenia and liver enzymes – markers disease monitoring

- **Rathakrishanan et al 2014. Clinical and Immunological Markers of Dengue Progression in a Study Cohort from a Hyperendemic Area in Malaysia. PLoS ONE**

DENV evasion of the immune system

DENV fitness

Virus-induced



Mosquito factors

Age

Nutritional status

Autoimmunity

Other factors

Vascular permeability



Dengue Pathogenesis

Antibody-dependent enhancement

Cross-reactive memory cells

Skewed cytokine profiles



Immune mediated



Genetic factors

Study: HLA Associations with Dengue

- HLA-A and –B alleles in a cohort from University Malaya Medical Center (2005- 2008) (Appanna et al., 2010)
- Using the WHO 1997 classification:

Classification	No. Patients
DF	41
DHF	51

- HLA-A and –B alleles in a cohort from Ampang Hospital and HTAR, Klang, Malaysia (2010- 2011)
- Using the WHO 2009 classification

Classification	No. Patients
ND	11
DwoWS	30
DwWS	183
SD	3

HLA distribution and association (WHO 1997)

HLA-A allele frequencies (>5%)
 Dengue: A*01, A*02, A*11, A*24, A*33
 Control: A*01, A*02, A*03, A*11, A*24, A*33

HLA-B allele frequencies (>5%)
 Dengue: B*07, B*13, B*15, B*35, B*38, B*40, B*46, B*51, B*52
 Control: B*7, B*13, B*15, B*18, B*35, B*40, B*51, B*58

HLA association in DHF patients

	Cntrl(%)	DwWS(%)	OR	95 % CI	P value
Total Population					
A*03	9.47	1.96	5.23	1.19-23.02	0.015
Malay					
B*13	3.13	15.79	0.18	0.03-0.90	0.049

HLA distribution and association (WHO 2009)

Common HLA-A alleles (>5%)

DwoWS: A*01, A*02, A*11, A*24, A*33

DwWS: A*01, A*02, A*11, A*24, A*33

Control: A*01, A*02, A*03, A*11, A*24, A*33

Common HLA-B alleles (>5%)

DwoWS: B*13, B*15, B*18, B*35, B*38, B*40

DwWS: B*13, B*15, B*18, B*35, B*40, B*44, B*58

Control: B*7, B*13, B*15, B*18, B*35, B*40, B*51, B*58

Dengue without warning signs

	Cntrl(%)	DwoWS(%)	OR	95 % CI	P value
Total Population					
A*03	9.5	2.4	4.186	0.948 - 18.48	0.044
A*34	1.1	9.8	0.098	0.020 - 0.474	0.001
B*15	12.6	24.4	0.448	0.231 - 0.868	0.020
Chinese					
A*24	9.4	35.7	0.186	0.047 - 0.740	0.023

Dengue with warning signs

	Cntrl(%)	DwWS(%)	OR	95 % CI	P value
Total Population					
A*03	9.5	2.7	3.788	1.712 - 8.383	0.001
B*15	12.6	19.6	0.592	0.360 - 0.975	0.045
Malay					
A*03	9.4	1.2	8.241	2.001 - 33.950	0.003
Chinese					
A*24	9.4	24.3	0.323	0.118 - 0.879	0.037
Indian					
B*57	6.5	25.0	0.207	0.060 - 0.716	0.016

Dengue without warning signs vs. Dengue with warning signs

	Without WS-AF (%)	With WS-AF (%)	OR	95 % CI	P value
Total Population					
A*33	6.1	15.3	0.359	0.139 - 0.926	0.032
A*34	9.8	3.0	3.548	1.379 - 9.125	0.011
Malay					
A*34	13.0	4.5	3.128	1.152 - 8.489	0.028

T Cell Responses

IFN- γ Enzyme-Linked Immunospot Array (ELISpot)

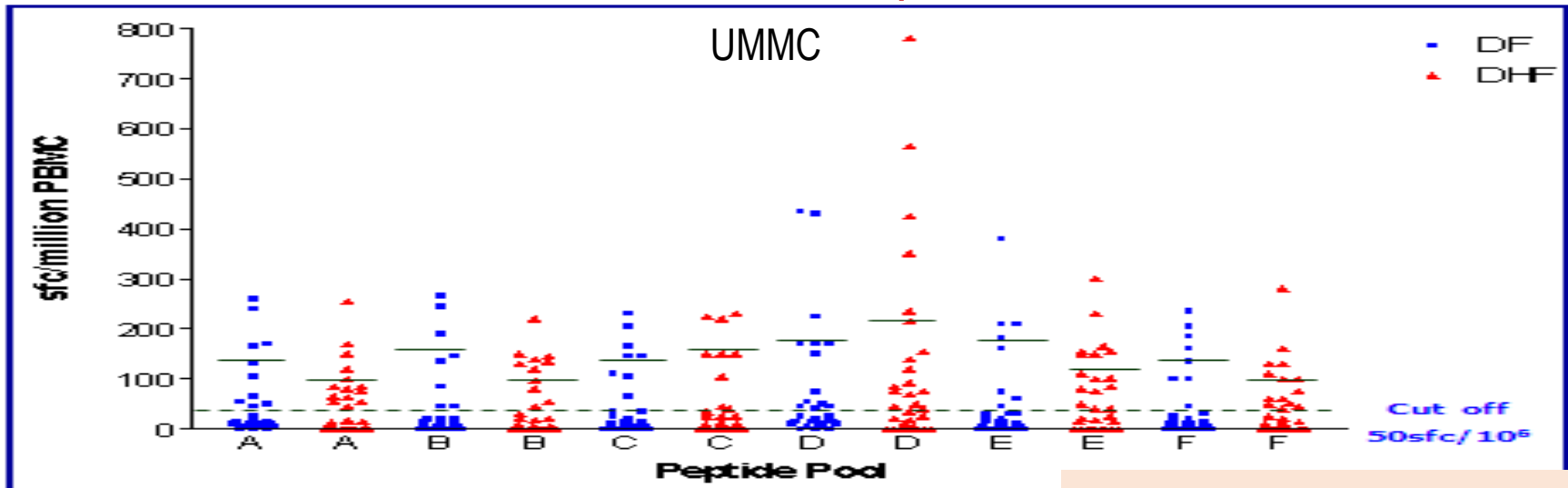
UMMC

- Peptide design:
 - DENV1 and DENV2-Indonesia, Thailand, Singapore
 - Against HLA-A*02, A*24, A*03, A*11, HLA-B*08 and B*27. –SYFPEITHI/RANKPEP
 - Initial: 252 peptides
 - Final: 32 peptides (top scoring)

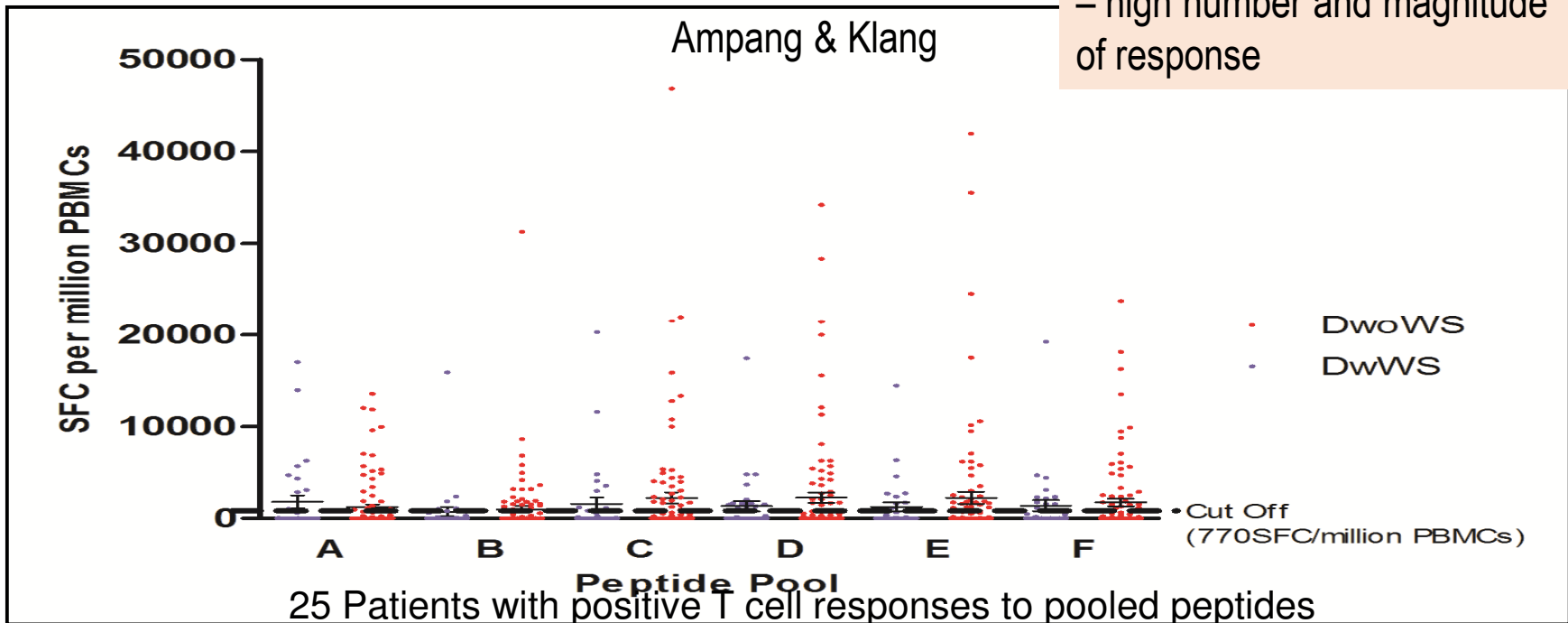
Ampang/Klang

- Peptide design:
 - DENV1-4 – Malaysia
 - Against HLA-A*03, A*11, A*24, B*18, B*4001, B*51, and B*53 from previous findings (Appanna et al., 2010) – SYFPEITHI/RANKPEP
 - Initial: 3369 peptides
 - Final: 36 peptides (binding score, DENV region, conservation amongst strains, whether it has previously been studied)

Pooled T cell responses

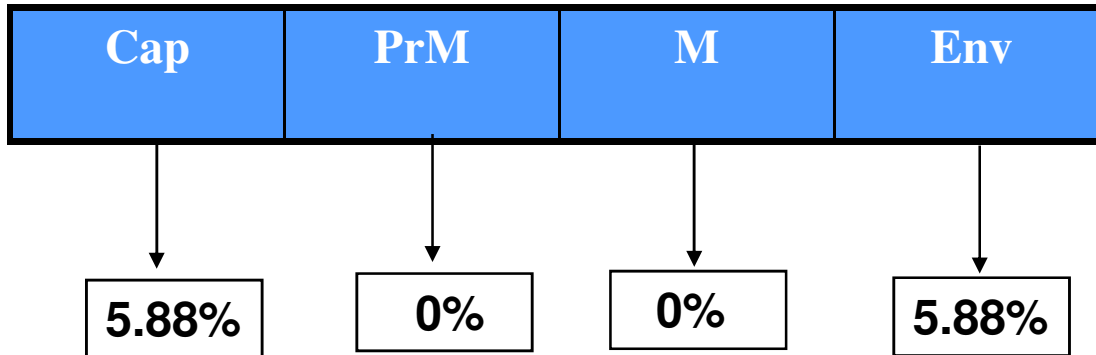


Non structural peptides
– high number and magnitude of response

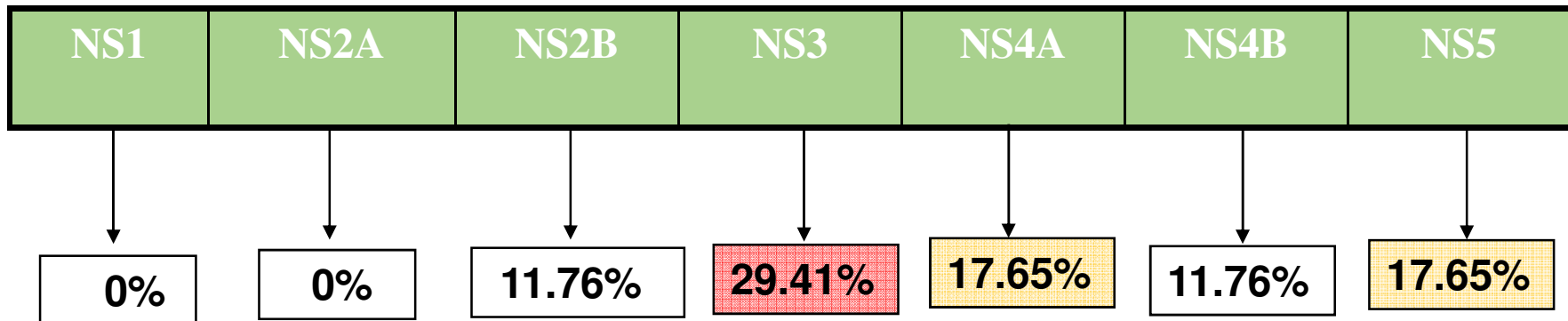


Individual T cell responses: UMMC

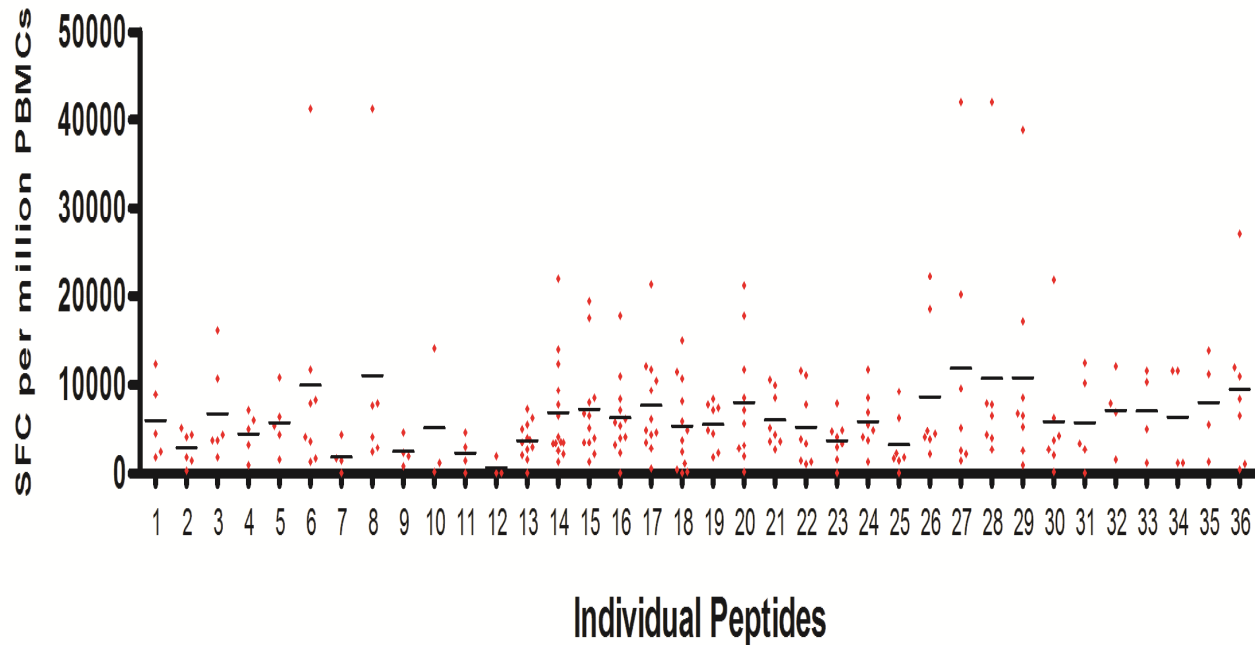
Structural region



Non Structural region



Individual T cell responses: Ampang & Klang



Peptide	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
No. Responders	3	1	4	1	3	4	1	3	1	1	1	0	2	8	9	7	9	6	4	7	6	4	4	6	3	5	5	5	7	3	2	2	1	1	2	4
Percentage (%)	33.3	11.1	44.4	11.1	33.3	44.4	14.3	42.9	14.3	14.3	14.3	0.0	14.3	57.1	64.3	50.0	64.3	42.9	40.0	70.0	60.0	40.0	40.0	60.0	33.3	55.6	55.6	55.6	77.8	33.3	25.0	25.0	12.5	12.5	25.0	50.0

NS1₁₈₇₋₁₉₅

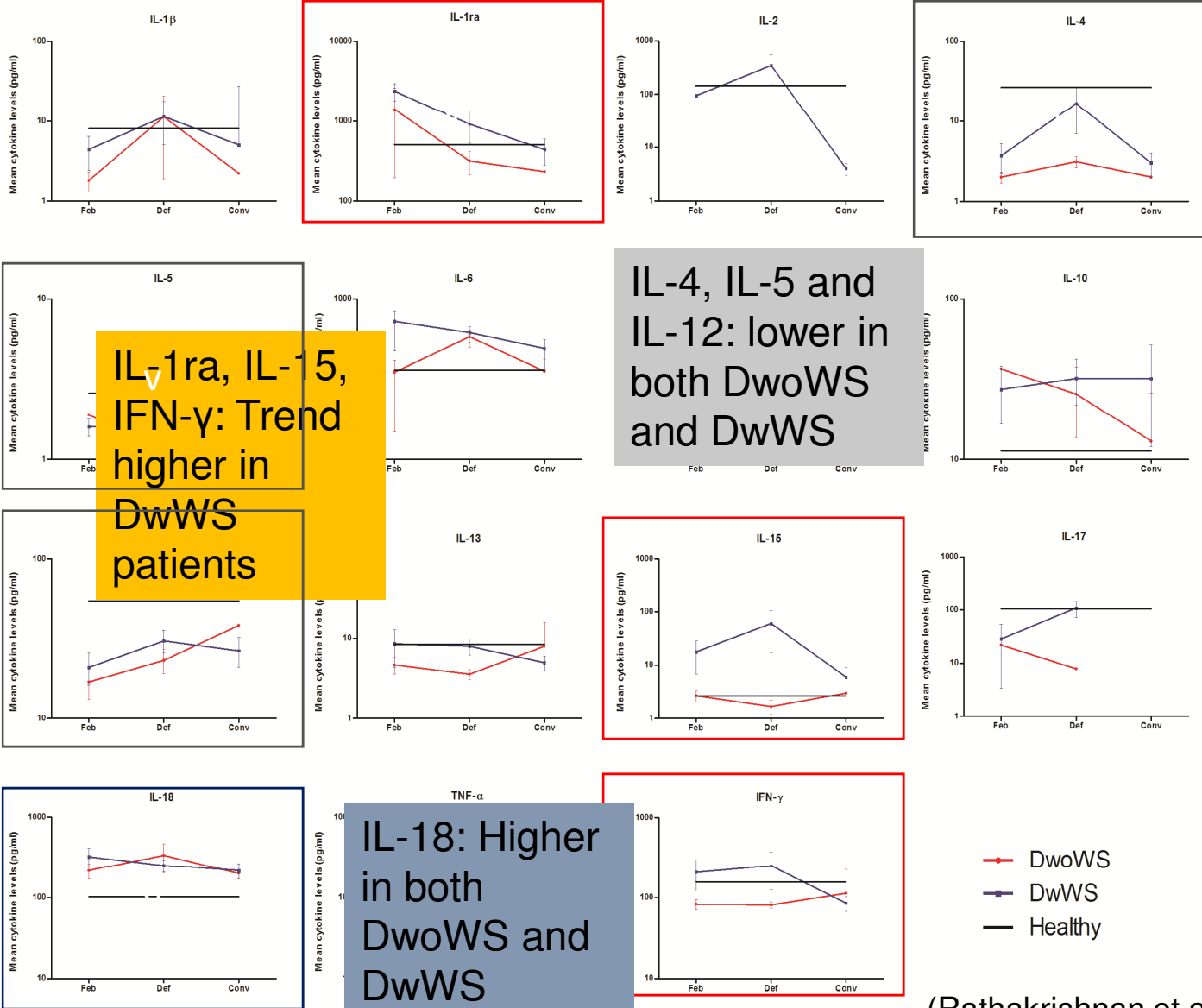
NS2A₁₇₉₋₁₈₈

NS peptides (NS4A, NS4B, NS5 and NS1)
 – elicited higher magnitude of response
 E peptide – 2 elicit ↑ response

Cytokine Expression in Dengue Patients

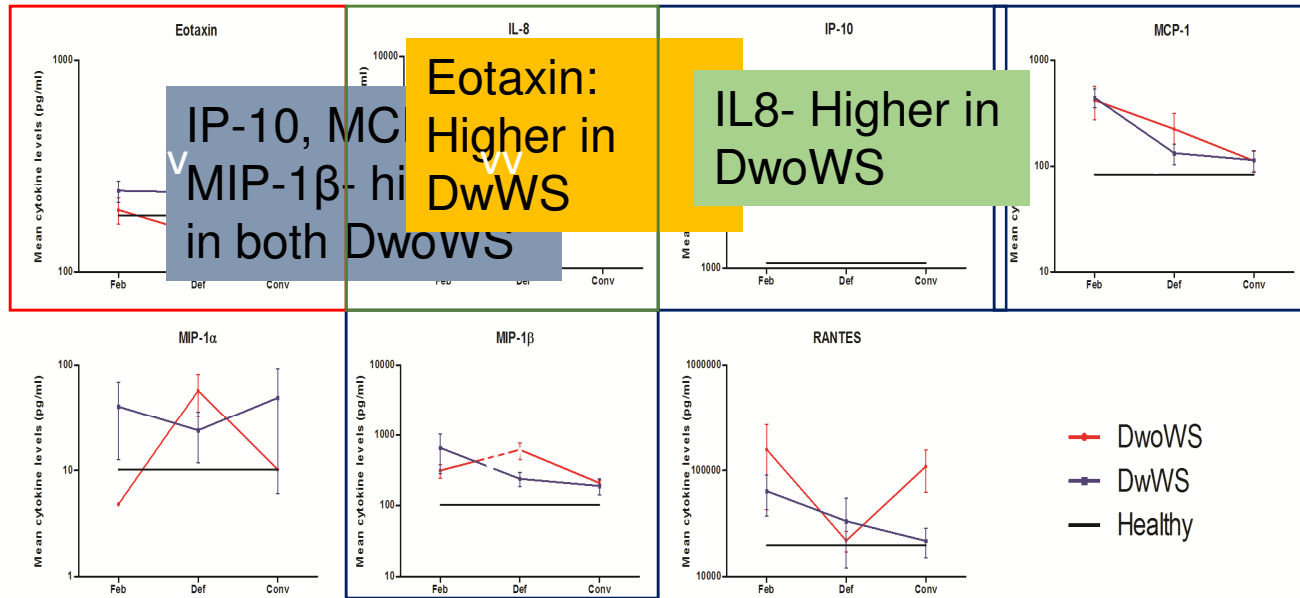
Multiplex Bead-based BioPlex Assay

A. Inflammatory Cytokines

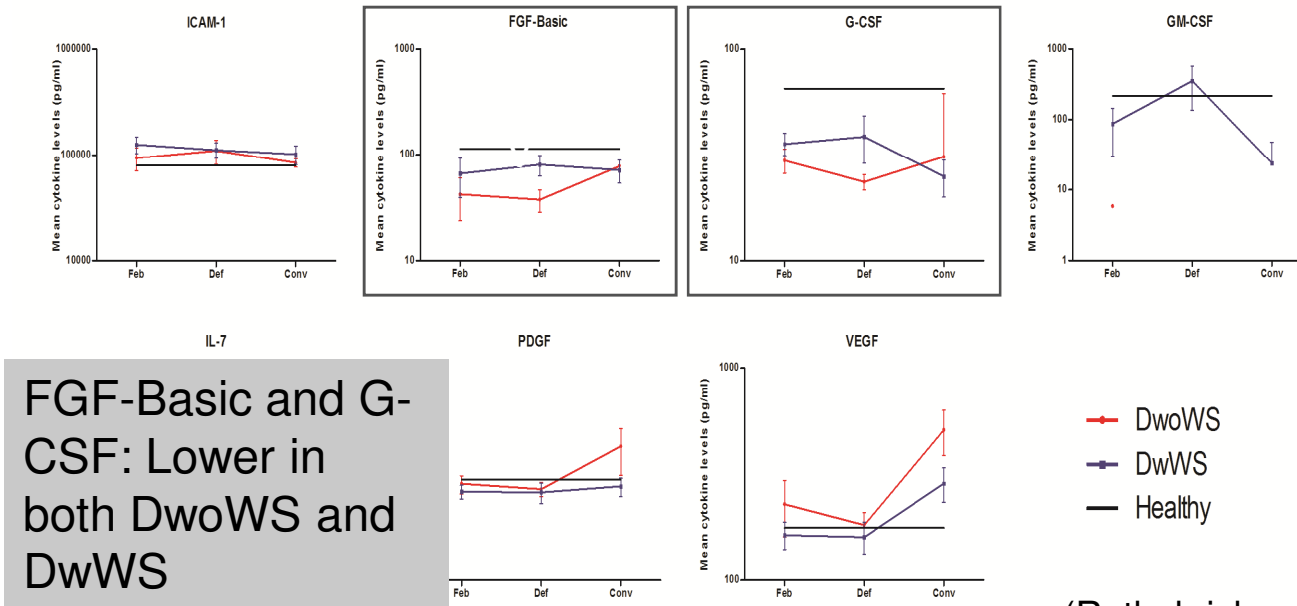


(Rathakrishnan et al., 2012)

B. Chemokines

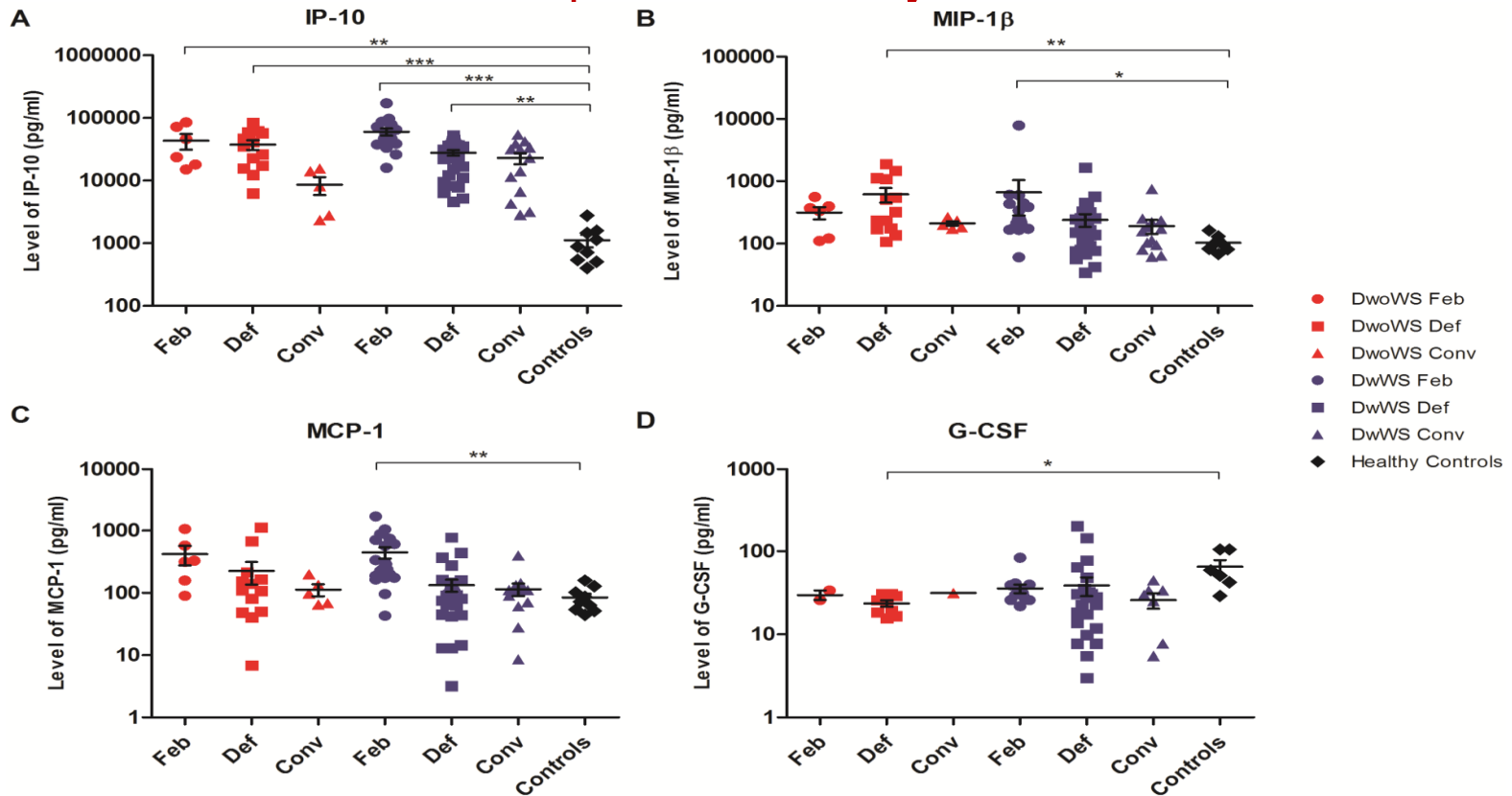


C. Adhesion Molecules and Growth Factors



(Rathakrishnan et al., 2012)

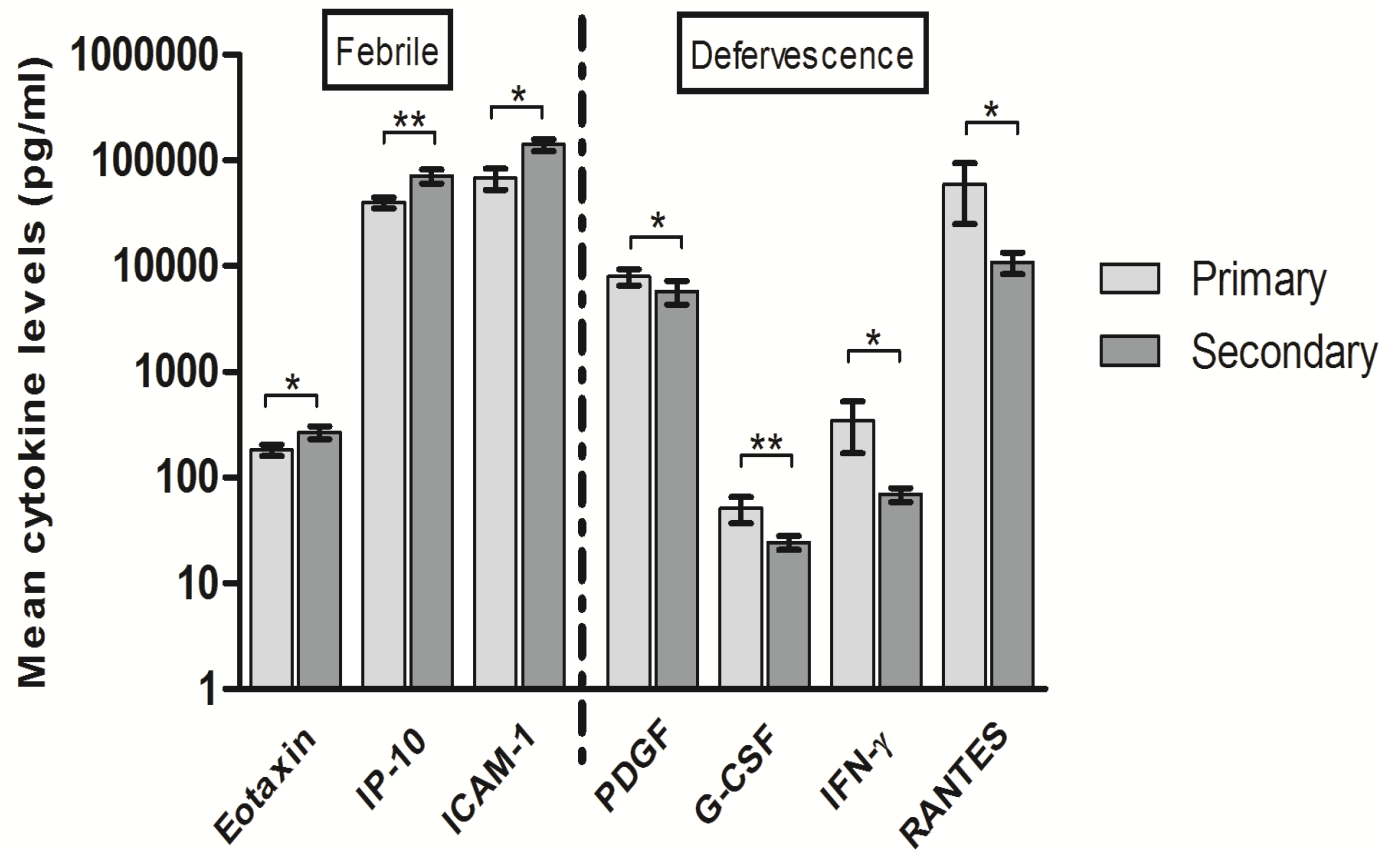
Significant cytokines at different phases of illness when compared to healthy controls



(Rathakrishnan et al., 2012)

Cytokines	Controls (pg/ml)	Acute (pg/ml)	Defervescence (pg/ml)	Conv
IP-10	1114.69 ± 250.45	DwoWS ↑↑ (43516.26 ± 12188.23) DwWS ↑↑ (58880.34 ± 7867.14)	DwoWS ↑↑ (35574.12 ± 6509.15) DwWS ↑↑ (26983.01 ± 3042.01)	-
MCP-1	84.11 ± 12.80	DwWS ↑↑ (438.87 ± 91.41)	-	-
MIP-1β	102.49 ± 12.53	DwWS ↑↑ (662.81 ± 380.06)	DwoWS ↑↑ (618.36 ± 163.05)	-
G-CSF	65.31 ± 13.16	-	DwoWS ↓↓ (23.56 ± 2.05)	-

Cytokine differences in primary and secondary infections (Rathakrishnan et al., 2012)



Correlation: Cytokines + Clinical Parameters

Febrile

1. ↓ Platelet
 - DwWS: IL-7, IL-12, PDGF
2. ↑ Aspartate aminotransferase (AST)
 - DwWS: ICAM-1, FGF-B, IL-13, IL-4, IL-12, VEGF

Convalescence

1. AST
 - DwoWS: MCP-1
 - DwWS: IFN-γ and IL-10

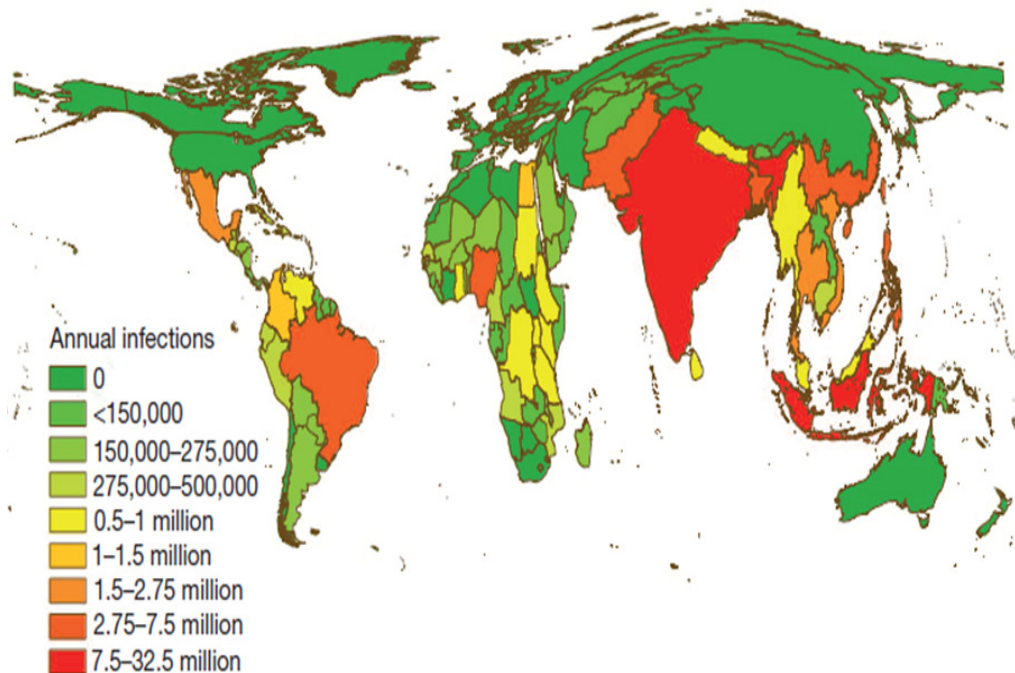
Defervescence

1. ↓ Platelet
 - DwoWS: VEGF, RANTES
 - DwWS: IL-5, IL-7, IL-12, PDGF, VEGF, RANTES
2. ↑ AST
 - DwoWS: IL-1ra, IL-10
 - DwWS: PDGF
3. ↑ ALT
 - DwoWS: IP-10

Black: Positive correlation
Red: Negative correlation

Dengue: Asymptomatic/Inapparent cases

- Asymptomatic > symptomatic
- ↑ importance: spread/contributor
- True roles of asymptomatic – unknown
 - Host protective factors?



390 million cases /year
Apparent: 96 million
Inapparent: 294 million

Study: Household members – Asymptomatic evaluation

62 household member from 58 dengue suspected patients

- Single sample collected (Whole blood, PBMC, plasma and serum)
- Diagnoses revealed
 - 3 confirmed
 - 14 presumptive

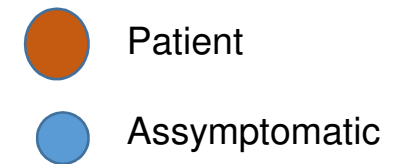
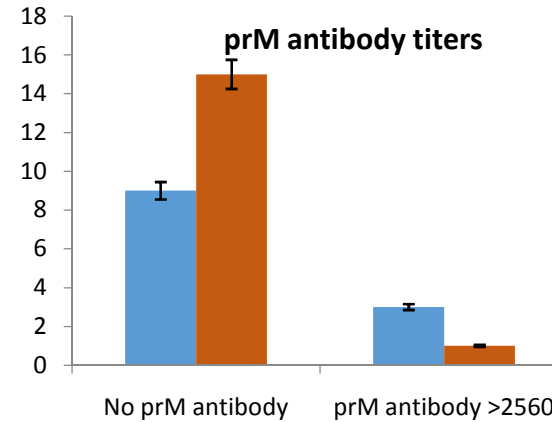
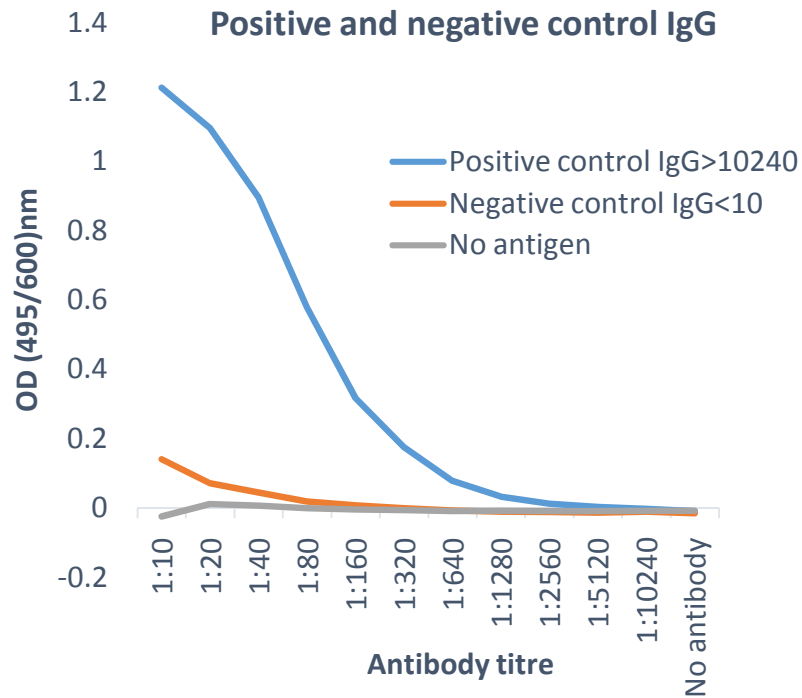
LABORATORY ASSAYS	Household Members
PCR + IgM	0
PCR + NS1	1
PCR + IgM + NS1	0
NS1 + IgM	2
IgM (PAIRED)	N/A
Sero-conversion	N/A
CONFIRMED DENGUE	3
IgM (SINGLE)	14
NS1 ONLY	0
PRESUMPTIVE DENGUE	14

Study: Laboratory Diagnostics for Asymptomatic Individuals

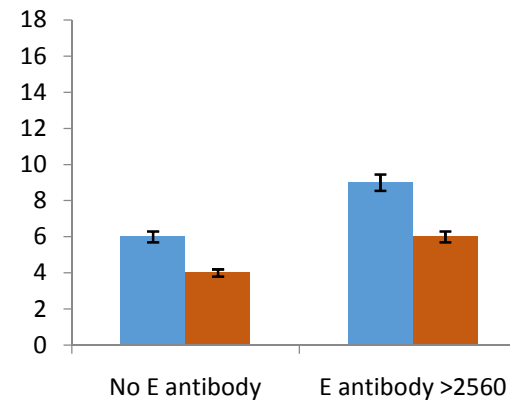
Plaque reduction neutralization test

Category	Monotypic Infection (%)	Polytypic Infection (%)	No neutralizing antibody detected (%)	Total
Acute-phase patient sera	17 (48.6)	16 (45.7)	2 (5.7)	35
Convalescent-phase patient sera	15 (42.9)	19 (54.3)	1 (2.8)	35
Asymptomatic household members	10 (28.6)	9 (25.7)	16 (45.7)	35
Total	42 (40.0)	44 (42.0)	19 (18.0)	105

prM and E antibodies: Patients vs. Asymptomatics



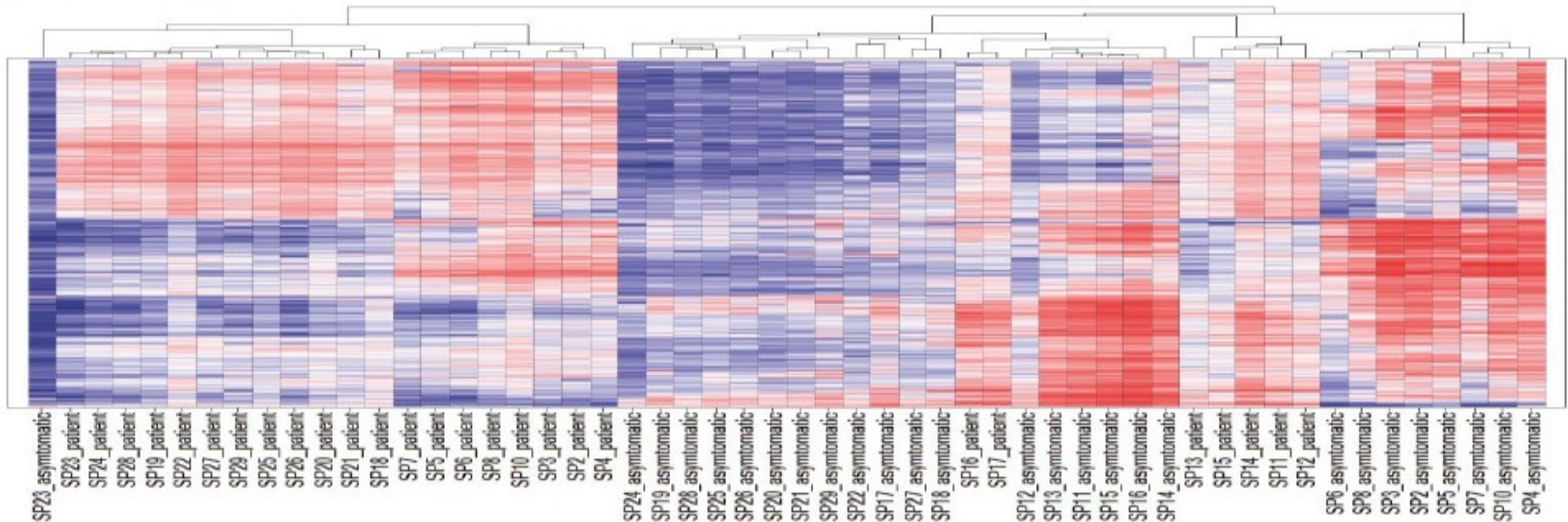
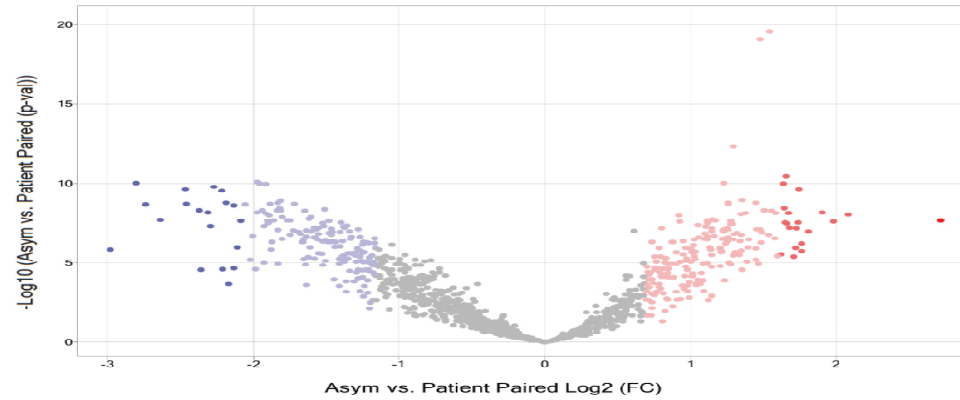
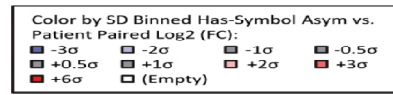
E antibody titers



- prM: Patients ↑ positivity (74%) vs. Asymptomatic (57%)
- E: Asymptomatic ↑ positivity (89%) vs. patients (83%)

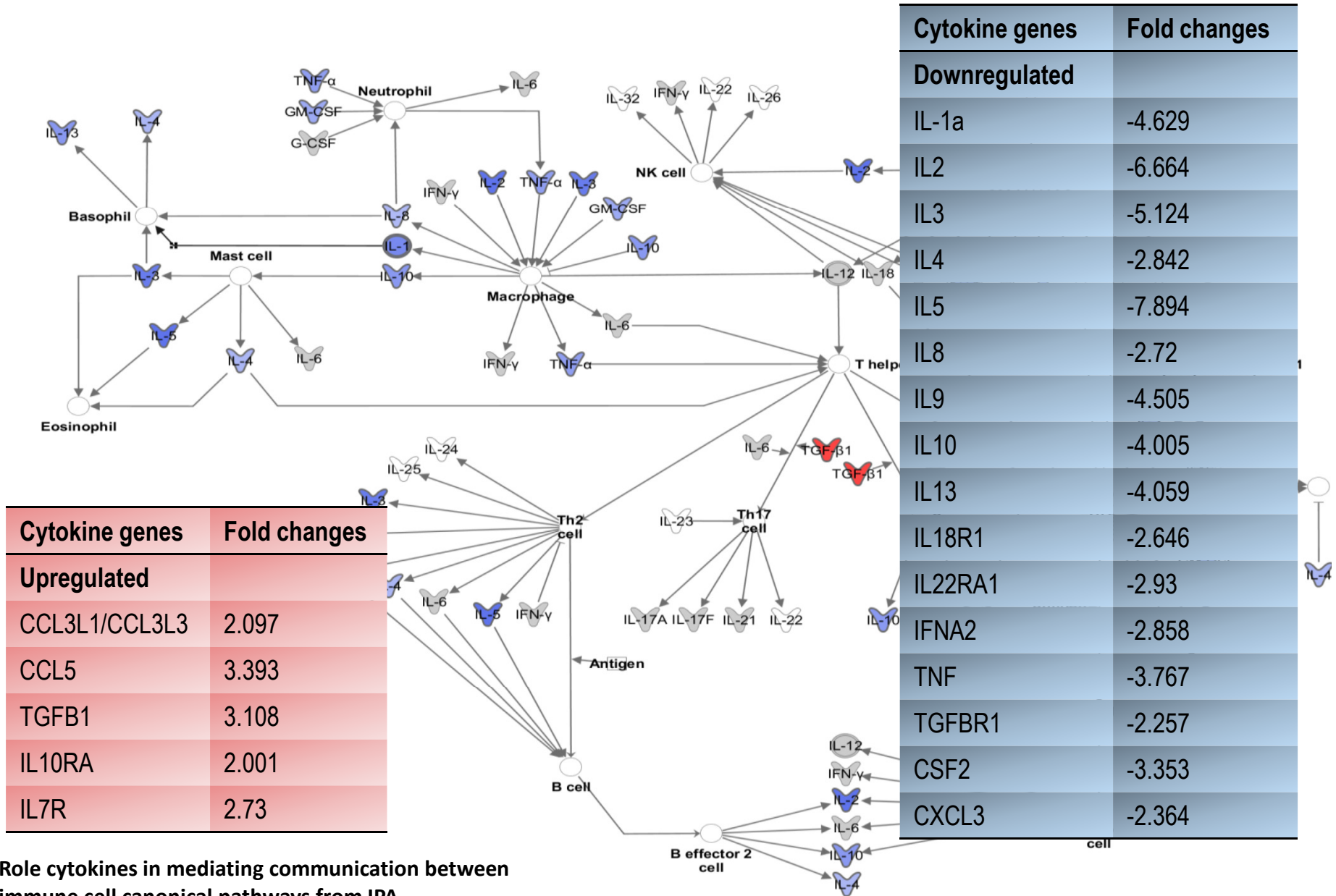
Asymptomatic vs Patients: Gene expression profiling

PBMCs of patient + household member pairs -- microarray analyses



Unsupervised hierarchical clustering discriminates symptomatic patients from asymptomatic individuals

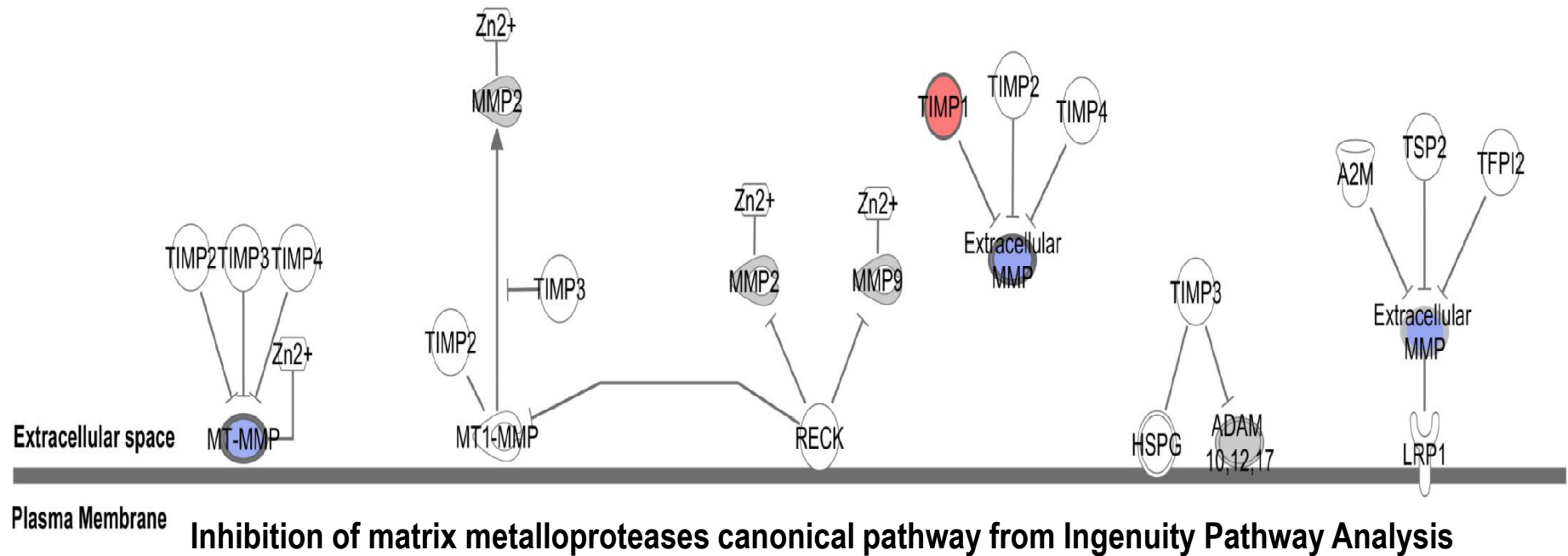
Cytokine involvement in asymptomatic infections



Role cytokines in mediating communication between immune cell canonical pathways from IPA.

Blue = down regulation; red = up regulation; grey = no change

MMP involvement in asymptomatic infections



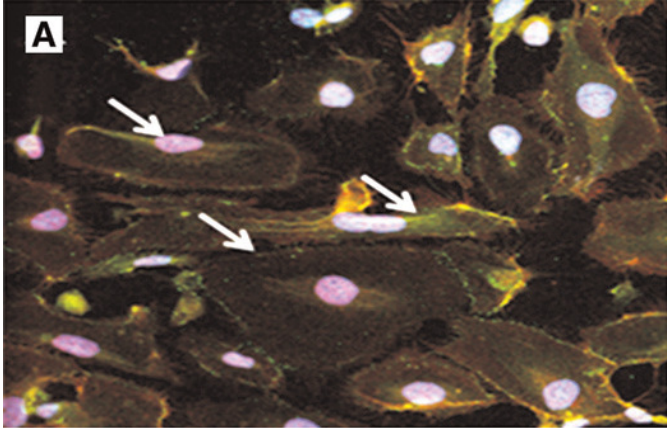
- **Dowregulated genes**

- MT-MMP- MMP15, MMP16, and MMP24
- Extracellular MMP- MMP8, MMP10, and MMP12

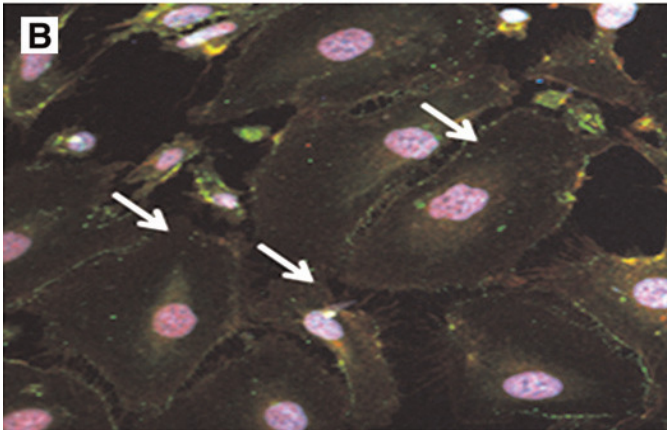
- **Upregulated genes**

- TIMP1 (inhibits activity of MMPs)

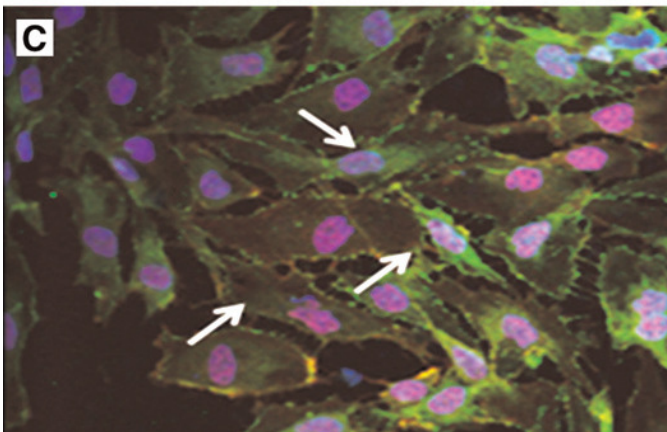
Patients serum vs. Pooled Rh-cytokines on HUVECs



Acute phase serum DHF patient

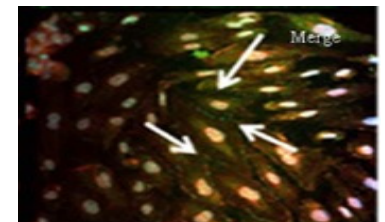


Defervescence phase serum DHF patient



Pooled recombinant cytokines: rhIP-10, rhRANTES, rhMCP-1, rhIL-1ra, rhEotaxin, rhIL-9

(Appanna et al., 2012)



Control

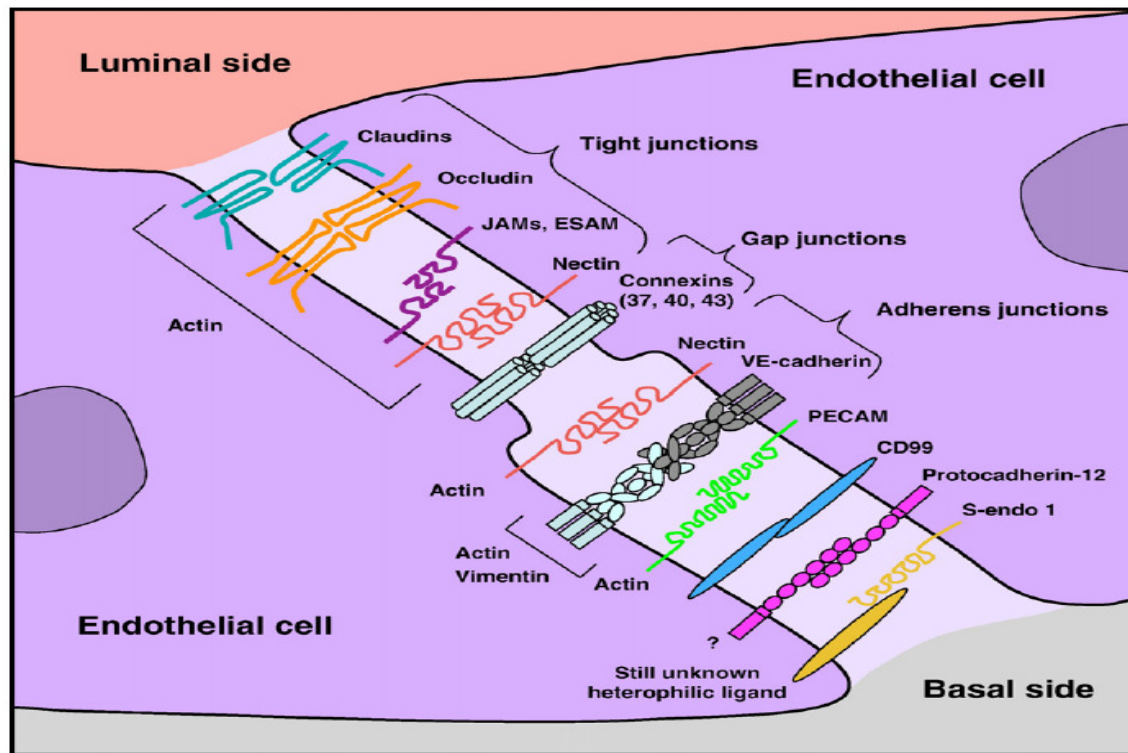
Effect of DENV and host immune system on ECs

Lee et al., 2006 on ZO-1: **MCP-1 ↑ endothelial permeability**

(i)DV2 (MOI 1), (ii)rhMCP-1, (iii)DV2 infected cell supernatant, (iv)DV2 infected cell supernatant treated with MCP-1 Abs

Kanlaya et al., 2009:

- 1) Actin : ↓
expression and disorganization
 - 2) PECAM-1: ↓
 - 3) ZO1 and VE-Cadherin: ↓
expression and redistribution
- DV2 (MOI 10)



Talavera et al., 2004 on Actin, Occludin, Vinculin and Phosphotyrosine, : **IL-8 ↑ endothelial permeability**

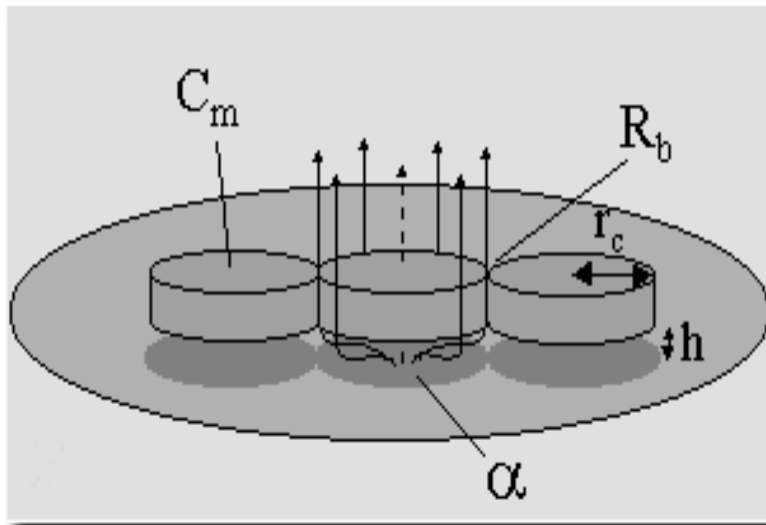
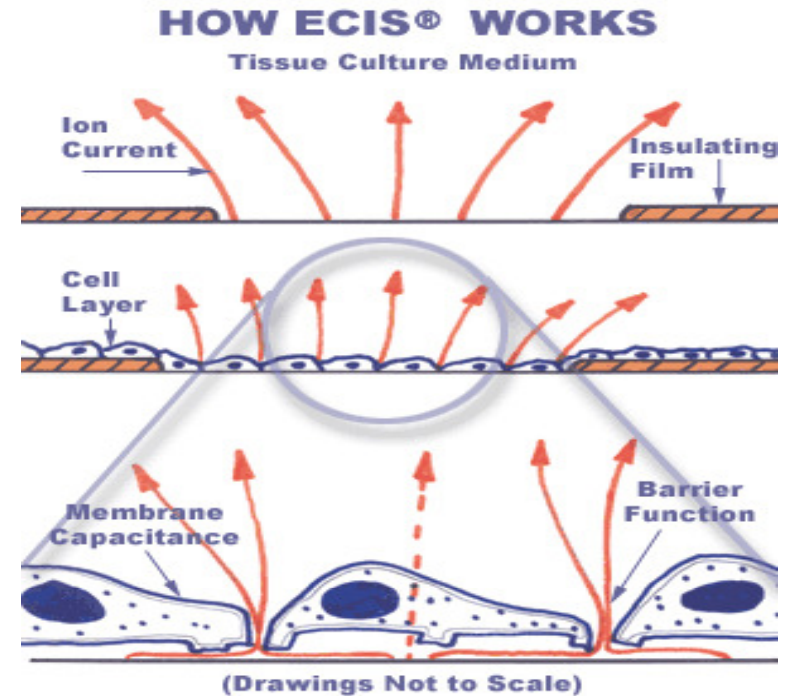
(i)DV2 (MOI 1), (ii)rhIL-8, (iii)DV2 infected cell supernatant, (iv)DV2 infected cell supernatant treated with IL-8 Abs.

Appanna et al., 2012 on ZO-1 and VE-Cadherin: **Cytokine partially play roles in ↑ endothelial permeability**

(i) rhIL-1ra, (ii)rhIL-9 (iii)rhMCP-1 (iv) rhRANTES (v)rhEotaxin, rhIP-10 (iv) DHF/DSS patients sera

Barrier Function Test : ECIS

- Electric Cell-Substrate Impedance Sensing (ECIS) technology
- Cells- act as insulators & Resistance \uparrow , when
 - cell no \uparrow
 - Cell morphology changes
 - nature of cell attachment.
- Treated ECs, (if) with functional changes, will change the impedance



ECIS Model

-valid when cells are confluent only

R_b – barrier resistance (paracellular)

α – impedance change beneath cells (cell-substrate)

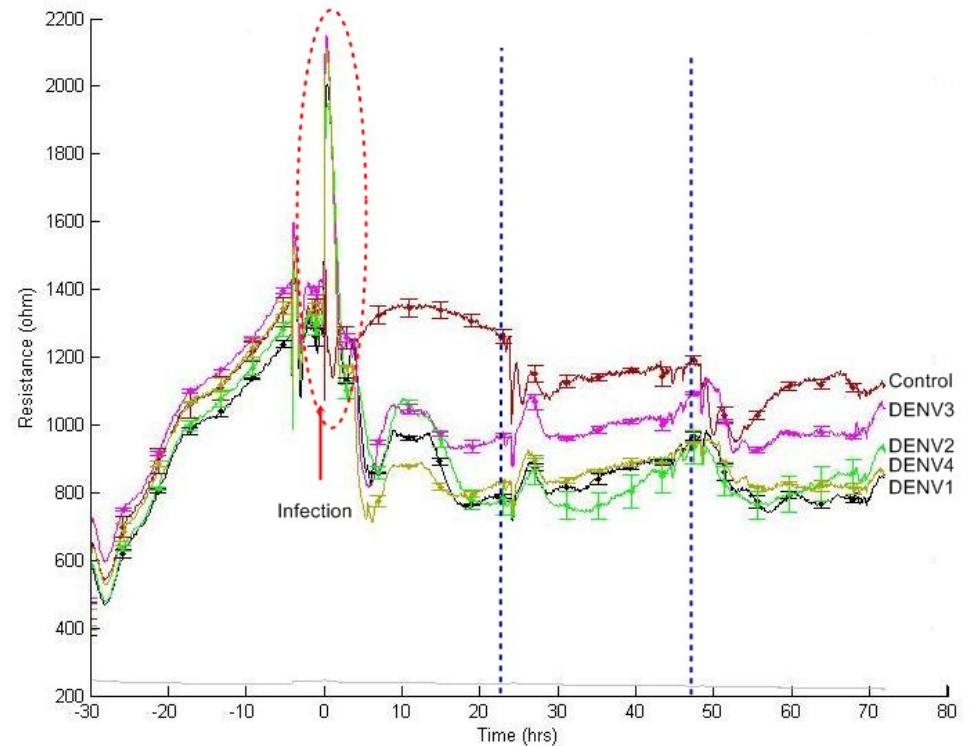
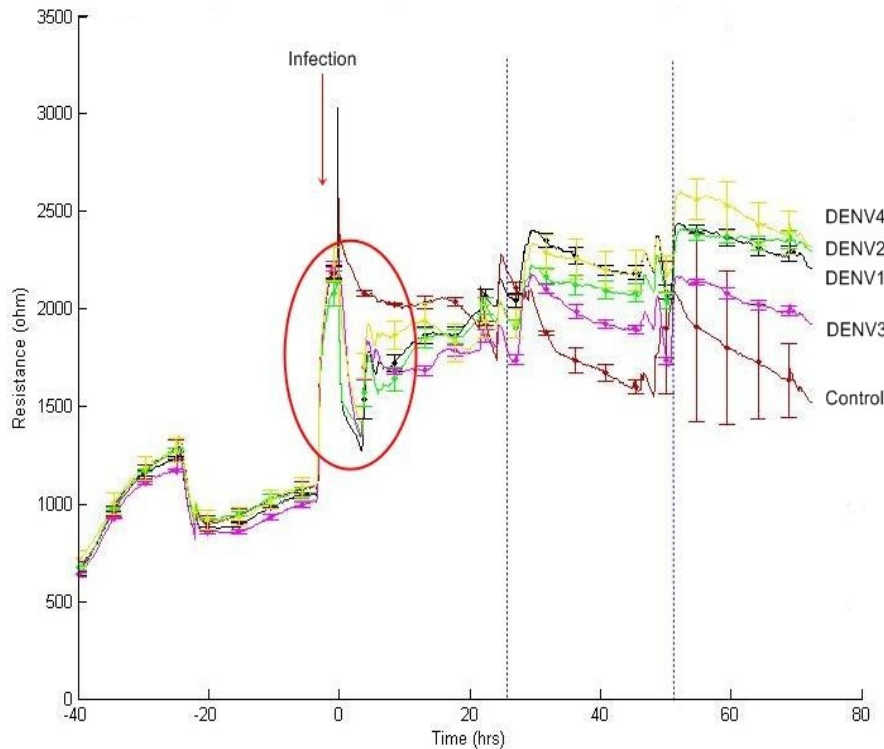
C_m – Membrane capacitance (transcellular)

h – distance between cells and electrode

r_c – radius of cells

Brain and Lung Microvascular Endothelial Cells

- At MOI 0.1 & 1.0: No significant difference in Resistance

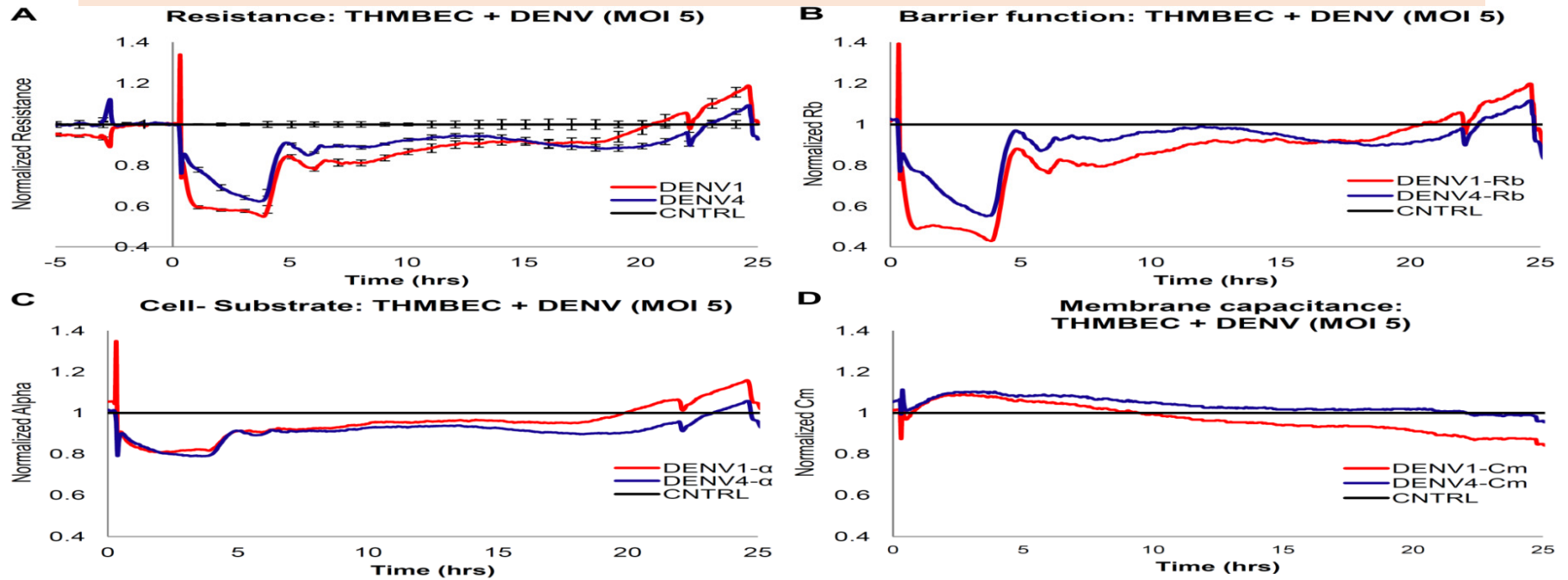


At MOI 5: Resistance ↓ upon DENV infection about 3-4hrs. R gradually ↑ and by 24hrs the barrier was tighter the non-infected control up to 72hr

At MOI 5: Resistance ↑ in DENV infected cells for about 1-1.5hrs and then drastically ↓ to below the control up to 72hr

Virus-modulated endothelial dysfunction

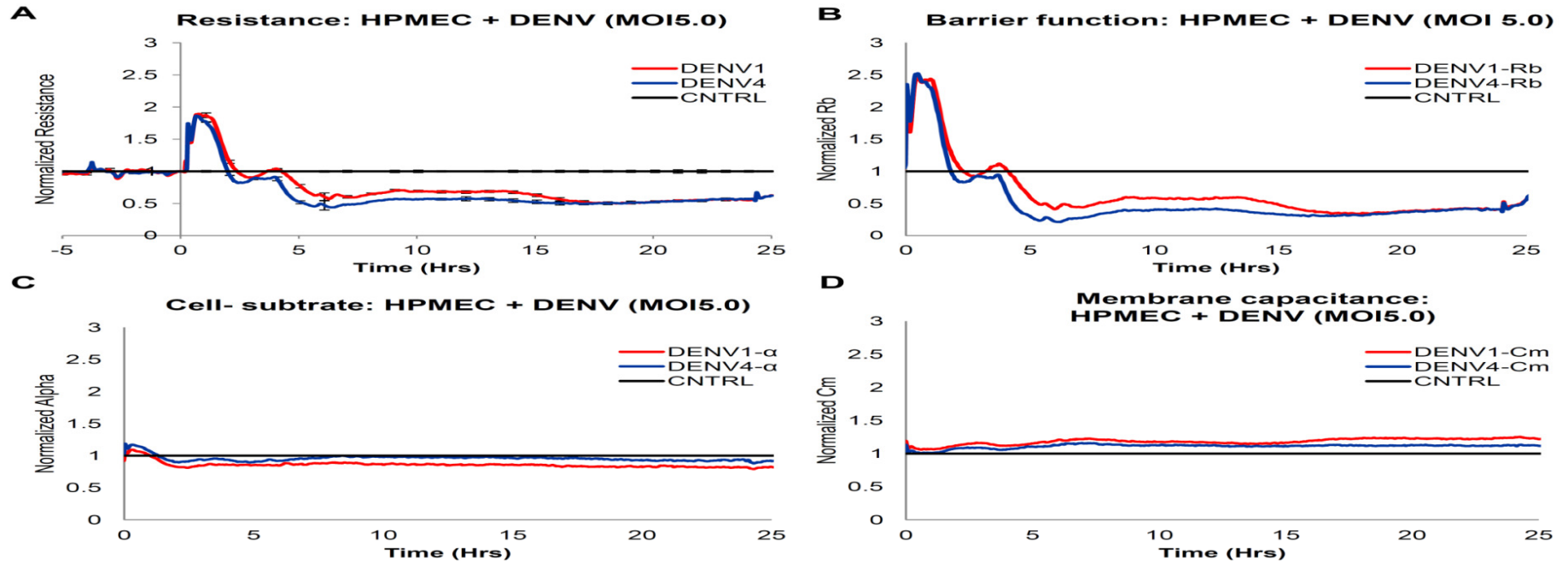
Brain microvascular endothelial barrier function: early DV infection (24h)



Junctional Protein	THBMEC-Control	DENV infected THBMEC					
		0h	1h	3h	6h	12h	24h
Tight Junction							
ZO1	Yes	↓↓↓	↓↓↓	↓↓	↓↓	↓	-
Occludin	Yes	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
Claudin-1	Yes	↑	↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
ESAM	ND	ND	ND	ND	ND	ND	ND
Gap Junction							
Cx43	ND	ND	ND	ND	ND	ND	ND
Adheren junction							
VE-Cadherin	Yes	-	↑	↓↓	↑↑↑	↑↑↑	↑
Inter-endothelial associated protein							
PECAM	Yes	↓	↓	↓	↓	↓↓	↓

Virus-modulated endothelial dysfunction

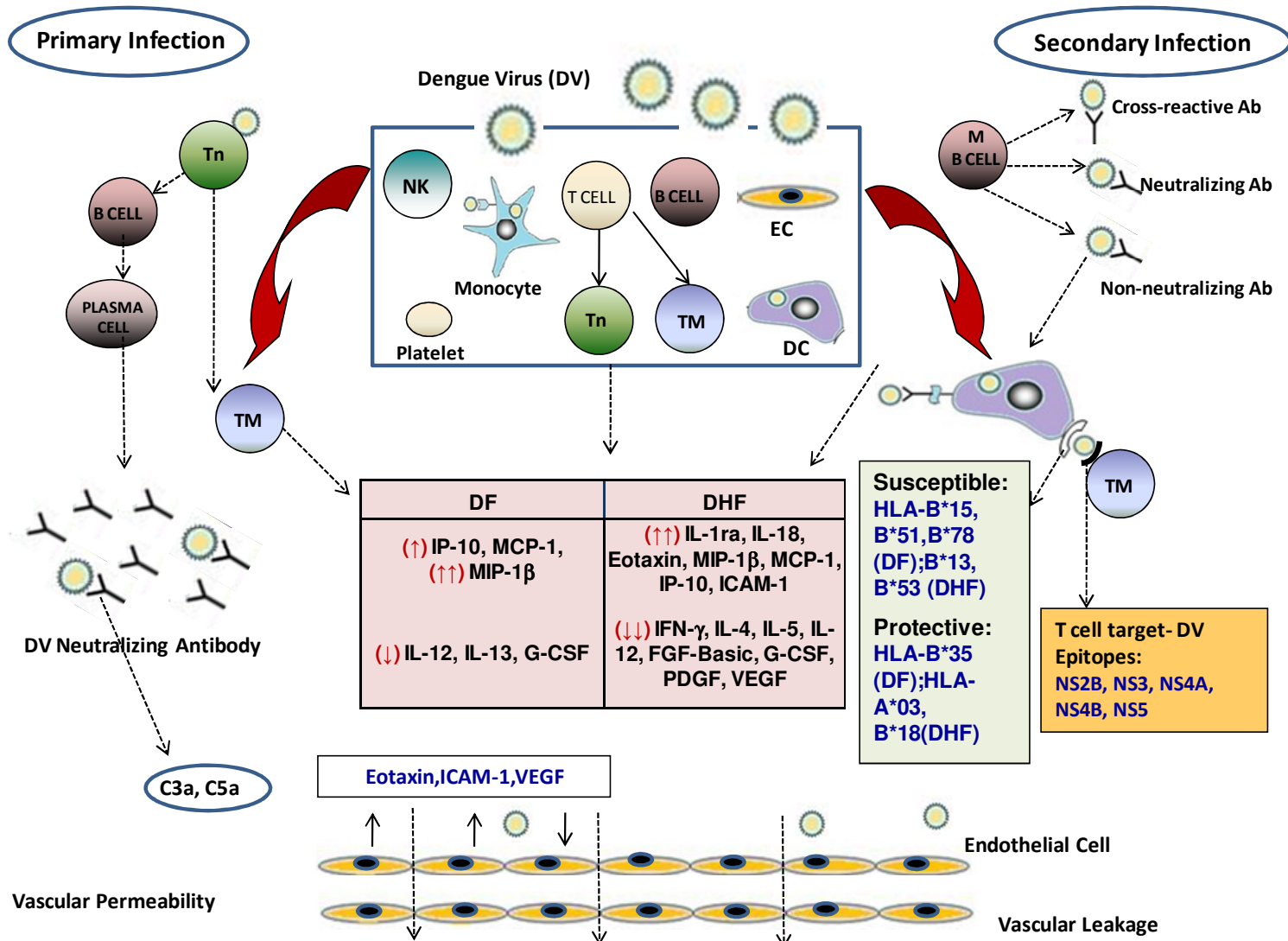
Lung microvascular endothelial barrier function: early DV infection (24h)



Junctional Protein	HPMEC-Control	DENV infected HPMEC					
		0h	1h	3h	6h	12h	24h
Tight Junction							
ZO1	Yes	↓	↓↓↓	↓↓↓	↓↓↓	↓↓	↓↓
Occludin	ND	ND	IND ↓	IND ↓	IND ↓	IND ↓	IND ↓
Claudin-1	ND	ND	ND	ND	ND	ND	ND
ESAM	Yes	↓↓↓	↓↓	↓↓	↓↓	↓↓	↓
Gap junction							
Cx43	ND	IND ↑	IND ↑	IND ↑	IND ↑	IND ↑	IND ↑
Adheren junction							
VE-Cadherin	Yes	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑	↑↑↑
Inter-endothelial associated protein							
PECAM	Yes	↓	↓	↓↓	↓	↓	↓

Summary

- DENV infection titer can affect ECs differentially
 - At MOI 0.1 and 1.0, no significant modulations observed
 - At MOI 5.0, immediate modulations to ECs
- DENV serotypes: despite the similar trends different serotype have different effect
 - Eg; DENV4 had minimal resistant change compared to the control
- ECIS modeling
 - The Rb (paracellular pathway) is most affected in both brain and lung ECs
 - In the infected BMEC, the cell-to-substrate adhesion was altered slightly
- Other cells being assessed : Dermal, retinal, hepatic and renal microvascular endothelial cells
- Assessment under sheer stress – ex-vivo with plasma/cells/virus samples from patients



95% of DHF occurs with secondary DV infection



UNIVERSITY OF MALAYA

The Leader in Research & Innovation



Dr. Jameela Sathar (Ampang Hospital)
Dr. Santha Kumari Natkunam (Klang Hospital)
Dr. Sasheela, Prof Lucy Lum and Dr. Rishya (UMMC)
Dr. Ramaprabha Appanna
Dr. Tang Yin Quan
Dr. Lee Sau Har
Anusyah Rathakrishnan
Adeline Yeo Syin Lian
Dr. Dennis Grab (JHU)
Dr. Anna Durbin (JHU)
Dr. Asif Khan (PUGSOM)

Financial support

- High Impact Research grants (University of Malaya)
(J-00000-73560 & H-20001-00-E000053)
- Postgraduate Research Funds grants (University of Malaya)
- Swami Institute for International Medical Education
(Johns Hopkins University)