Dysregulation of Complement Factor H During Dengue Virus Infection *in vitro*

By

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Thesis
Submitted to Flinders University
for the degree of

Doctor of Philosophy

College of Medicine and Public Health
February 2023

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Abstract

Dengue Virus (DENV) infects over 390 million people annually, causing debilitating and potentially life threatening disease. Yet, there is currently no approved antiviral to treat dengue and only one vaccine with limitations on its utility. Severe dengue can be fatal, and is caused by an exaggerated inflammatory response, driven partially by dysregulated complement activation, which correlates with poorer patient outcome. Previously, our laboratory has demonstrated in vitro that excessive complement alternative pathway (AP) activation in response to DENV results from increased factor B (FB), despite induction of mRNA for the AP regulator factor H (FH). Therefore, this project aimed to define the stimuli of FH induction, quantitated FH mRNA and protein and characterised FH-ligands and FH-cell localisation in response to DENV infection.

In Chapter III, changes in abundance of mRNA for a panel of AP proteins were analysed *in silico* using publicly available microarray data from whole blood samples taken from dengue patients. Analysis revealed FH, and AP activator Factor B (FB), were highly increased during acute infection and that increases in FB were disproportionately higher than FH. These findings are consistent with our group's previous published data from DENV-infected cells *in vitro*, validating that similar responses to DENV-infection may occur for patients. Next, *in vitro* models were used to study the mechanisms driving FH mRNA and protein production during DENV-infection further. Previous characterisation of complement proteins during DENV-infection *in vitro* have been performed in primary macrophages and endothelial cells. These models suffer from heterotypic responses due to inter-donor variability and are difficult to acquire. Therefore, a new model for investigating complement during DENV-infection was sought. In Chapter IV, HeLa were selected as a model for FH mRNA and protein production in response to DENV, as they recapitulate increased FH mRNA but unchanged FH protein in the extracellular space. Using HeLa, it was demonstrated DENV induced full length FH mRNA and protein. Additionally, changes in FH ligands, heparan sulphate (HS) and sialic acid, were quantitated and were increased and unchanged on the cell membrane, respectively. Digestion of cell surface HS released significantly more FH from DENV, however, concomitant digestion of cell surface HS and sialic acid did not increase the release of FH further. Thus FH on DENV infected cells favoured HS binding which was not significantly impacted by the presence of sialic acid. It was then revealed using confocal microscopy that FH was retained intracellularly in response to DENV, which provided an explanation for discordant mRNA and extracellular FH protein *in vitro.* Interestingly, retention of FH was also observed for uninfected bystander cells in the DENV-

infected culture. This formed a rationale for investigating induction of FH *in trans* by the secreted proteins produced from DENV-infected cells. In Chapter V, the supernatant collected from DENV-infected HeLa was size-filtered and used to assess FH mRNA and intracellular protein induction. Analysis by qPCR and immunofluorescent microscopy revealed that large (>50 kDa) secreted proteins induce FH mRNA, but small (<50 kDa) secreted proteins induce FH-cell localisation. Mass spectrometric analysis of HeLa supernatant revealed increased abundance of many complement proteins in response to DENV-infection. Specifically, the >50 kDa supernatant fraction of DENV infected cells contained highly elevated (C4 and alpha-2 macroglobulin) and uniquely detectable (C5 and FB) complement proteins but reduced relative FH. Cytokine array also revealed the elevation of IL-6 and IL-8 in the <50 kDa fraction of DENV-infected HeLa supernatant. These results suggest dual-mechanisms for FH mRNA production and protein retention. FH mRNA induction may be caused by DENV or large complement proteins, however, small, non-viral, secreted proteins such as cytokines induce FH-cell association. These findings therefore advance our understanding of processes that direct FH induction and localisation during DENV-infection and potentially other viral infections more broadly.

In addition to investigations of complement regulatory proteins during DENV-infection, a literature review was performed in Chapter VI to collate and critically evaluate the prevalence of complement-associated diseases in Indigenous Australians. The prevalence of these diseases are affected by the deletion of two complement genes (*CFHR3-1Δ*) and were compared to other vulnerable people groups possessing this deletion. The data are in support of the hypothesis that the complotype of people of Indigenous Australian descent contributes to disparate disease prevalence compared to non-Indigenous Australians. The review establishes a precedence for investigating polymorphisms in the Indigenous Australian complotype which could ultimately inform a tailored approach to treating infectious and autoimmune diseases in Indigenous Australian patients in the future. The literature review within outlines a potential therapeutic avenue to help close the gap in disease disparities of Indigenous Australians by targeting complement. Complement regulation is a vital component of a healthy immune system, both in response to infection and in controlling autoimmune diseases. Additionally, previously overlooked connections between disease prevalence in Indigenous Australians and complement have now been established. Thus, this thesis outlines the role of complement regulatory proteins in both autoimmune and infectious diseases, defining targets of the complement system as potential therapeutic avenues during disease.

Declaration

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed Joshua Gregory Dubowsky

Date 22/07/2022

Acknowledgements

I am very thankful for the opportunity afforded to me by Flinders University and the support I have received from all of its staff members and my fellow students.

None of this would have been possible without the years of support and guidance from my supervisor Professor Jillian Carr. I am very thankful to have developed such a valuable relationship with Jill, which has shaped me into the scientist I am today. I would also like to thank Dr Binoy Appukuttan for his role as my co-supervisor. His support, perspectives, advice, and jokes were always greatly appreciated.

Thank you to the many scientists who helped me to develop and understand the concepts important to this thesis. Especially to Dr Sheila Cabezas, Dr Nicholas Eyre, Professor David Gordon, Professor Jamie Craig, Professor Alex Brown, and Mr Jose Estevez. I would also like to extend this thank you to the staff at Flinders who took the time to teach me the techniques used for this project. Especially to Ms Nusha Chegeni, Ms Kamelya Aliakbari, Mrs Pat Villimas, Dr Lauren Jones, and Professor Tim Chataway.

Thank you to Professor Michael Beard and Dr Michael Jackson for their support during my candidature and feedback on my work as it developed.

Thank you to the Carr lab members, Mr Luke Kris, Ms Evangeline Cowell, Ms Hawraa Jaber, Mrs Valeria Sanders, Mr Timothy Penn, Ms Amy Green and Dr Roman Lenchine. Your support, company, conversations, and critique have been invaluable.

I am grateful for the opportunity provided by the Australian Government who financially supported my candidature through the Australian Government Research Training Program Scholarship.

Finally, I would like to thank my family for their support and love. To my wife Megan, we both know that your hard work, patience, and sacrifice have been vital during this project. To my son Leo, who can always make me smile, I hope I might instil the wonders of science in you as my father did for me.

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Abbreviations

ADE	Antibody Dependent Enhancement
aHUS	Atypical Haemolytic Uraemic Syndrome
ALT	Alanine Aminotransferase
AMD	Age Related Macular Degeneration
ANOVA	Analysis Of Variance
AP	Alternative Pathway
AST	Aspartate Aminotransferase
AU	Arbitrary Units
BHK	Baby Hamster Kidney
BSA	Bovine Serum Albumin
CCP	Complement Control Protein
cGAMP	Cyclic Guanosine Monophosphate–Adenosine
cGAS	Cyclic GMP-AMP Synthase
CNS	Central Nervous System
CP	Classical Pathway
CR	Complement Receptor
CRIg	Complement Receptor Immunoglobulin
CRISPR	Clustered Regular Interspersed Short Palindromic Repeat
CRP	C-Reactive Protein
CSF	Cerebral Spinal Fluid
CTSL	Cathepsin-L
DAF	Decay Accelerating Factor
DC	Dendritic Cell
DC-SIGN	DC-Specific Intercellular Adhesion Molecule-3 Grabbing Nonintegrin
DENV	Dengue Virus
DMEM	Dulbecco's Modified Eagle Medium
DMMB	Dimethyl Methylene Blue
dNTP	Deoxynucleotide Triphosphate
DPBS	Dulbecco's Phosphate
EC	Endothelial Cell
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
ESKF	End Stage Kidney Failure
FB	Factor B
FC	Fragment Crystallisable
FCS	Foetal Calf Serum
FD	Factor D
FDA	Food And Drug Association
FH	Factor H
FHL-1	Factor H Like-1
FHR	Factor H Related
FI	Factor I
GAG	Glycosaminoglycan
GEO	Gene Expression Omnibus

GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Guanosine Monophosphate
GP	Glycoprotein
GSE	Gene Expression Omnibus Series
GWAS	Genome-Wide Association Study
HEK	Human Embryonic Kidney
HGSNAT	Heparan-A-Glucosaminide N-Acetyltransferase
HIV	Human Immunodeficiency Virus
HNF	Hepatic Nuclear Factor
HPSE	Heparanase
HRP	Horse Radish Peroxidase
HS	Heparan Sulphate
HSPG	Heparan Sulphate Proteoglycan
HUVEC	Human Umbilical Endothelial Cells
IF	Immunofluorescence
IFITM	Interferon-Induced Transmembrane
IFN	Interferon
IFNAR	Interferon A/B Receptor
IL	Interleukin
IRF	Interferon Regulator Factor
ISG	Interferon Stimulated Gene
ISRE	Interferon Stimulated Response Element
ITIH	Inter-Alpha-Trypsin Inhibitor-4
IU	International Unit
JAK	Janus Kinase
JEV	Japanese Encephalitis Virus
LB	Luria-Bertani
LPS	Lipopolysaccharide
MAC	Membrane Attack Complex
MASP	MBL Associated Serine Proteases
MAVS	Mitochondrial Antiviral Signalling Protein
MBL	Mannose Binding Lectin
MDA	Malondialdehyde
MDA5	Melanocyte Differentiation-Associated Protein 5
MDM	Monocyte Derived Macrophage
MITF	Melanocyte Induced Transcription Factor
MOI	Multiplicity Of Infection
MWCO	Molecular Weight Cut Off
NCBI	National Centre For Biotechnology Information
NEB	New England Biolabs
NEU	Neuraminidase
NF-κβ	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NKT	Natural Killer T-Cell
NLRD	Notifiable Low Risk Dealing
NS	Non-structural

PAGE	Polyacrylamide Gel
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Syndrome
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
RCA	Regulators Of Complement Activation
RIG-I	Retinoic Acid Inducible Gene - I
RPM	Rotations Per Minute
RT	Reverse Transcription
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
SCR	Short Consensus Repeat
SEM	Standard Error Of The Mean
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
STAT	Signal Transducer And Activator Of Transcription
STING	Stimulator Of Interferon Genes
TANK	TRAF Family Member-Associated NF-Kappa-B Activator
TBA	Thiobarbituric Acid
TBE	Tris Base, Boric Acid And EDTA
TBK	Tank Binding Kinase
TBST	Tris Buffered Saline With Tween 20
TF	Transcription Factor
TGA	Therapeutics Good Association
TIM/TAM	T Cell/Transmembrane, Immunoglobulin, And Mucin/Tyro3, Axl, And Mer
TLR	Toll-Like Receptor
TNF-	Tumour Necrosis Factor
TRAF	Tumour Necrosis Factor Receptor-associated factor
UTR	Untranslated Region
UV	Ultraviolet
WHO	World Health Organisation
WNV	West Nile Virus
WT	Wild Type
ZIKV	Zika Virus

Publications and Presentations

Accepted peer reviewed publications:

Jillian M. Carr, Sheila Cabezas-Falcon, **Joshua G. Dubowsky**, Jarrod Hulme-Jones, David L. Gordon. Dengue virus and the complement alternative pathway. FEBS Lett. 2020 Aug; 594(16): 2543-2555. doi: 10.1002/1873-3468.13730. Epub 2020 Jan 24. PMID: 29743365

Manuscripts in progress

Joshua G Dubowsky, Jose J Estevez, Binoy Appukuttan, Alex Brown, Jamie E Craig, Jillian M Carr. Disease profiles in the Indigenous Australian population are suggestive of a common complement control haplotype. (Under Review)

Joshua G Dubowsky, Binoy Appukuttan, David L Gordon, Jillian M Carr. Dengue Virus Infection Induces Factor H Through Large Secreted Molecules and Intracellular Complement.

Presentations

Joshua G. Dubowsky, Binoy Appukuttan, Jillian M. Carr. Complement Factor H expression in Dengue Virus Infected Cells. Australian and New Zealand Society for Immunology, ASI 2019, Adelaide Australia, December 2019. (Poster Presentation)

Joshua G Dubowsky, Jillian M Carr. Dengue Infection Causes Membrane Recruitment of Factor H Through Ligands other than Glycosaminoglycans. Lorne Infection and Immunity 2021. Attended online. February 2021.

Joshua G Dubowsky, Binoy Appukuttan, Jillian M Carr. Dengue Virus Infection Induces Factor H production, Relocation to the Nucleus and binding to cell surface Heparan Sulphate. International Complement Society, 28th International Complement Virtual Workshop. Hosted online. December 2021. (Poster Presentation)

CHAPTER I

INTRODUCTION

I-1 Dengue Virus

I-1.1 Global impact of Dengue virus

Dengue is the most important, prevalent, and rapidly spreading arboviral disease today and is modelled to infect more than 390 million people per year.[1, 2] Dengue is the disease caused by Dengue virus (DENV) infection which typically presents with self-limiting but debilitating symptoms.[3] It is difficult to estimate the true number of total DENV infections for many reasons, including underreporting and asymptomatic infections.[2] A significant factor in the startlingly high infections per annum is that greater than half of the global population live in areas where the mosquito *Aedes aegypti*, the primary transmission vector, can be found.[4] The temporarily incapacitating symptoms combined with the number of those infected, incur substantial costs estimated to range from $9-40 billion USD annually (as of 2016).[2, 5] Furthermore, after the virus is no longer detectable within patient blood, 'severe dengue' can develop as a result of an excessive immune response.[3] Severe dengue requires immediate medical attention and symptom management, which can be difficult to access in the resource poor settings that represent many dengue endemic countries.[6, 7] As no antivirals or therapeutics have been approved for severe dengue, a need persists for DENV research that aims to address the mechanisms of severe disease and to define therapeutic targets.

I-1.2 Clinical manifestations of dengue

Dengue symptoms typically present 4-8 days following the bite of a DENV-infected mosquito and outcomes of infections can range from asymptomatic to life threatening severe dengue.[3] Typical clinical presentations include fever, myalgia, arthralgia, intraocular pain, gastric discomfort including vomiting and diarrhoea, rashes and fatigue which resolve within a week for 99% of infections.[3] A hallmark of dengue is vascular leakage, resulting in petechiae and a blanching rash.[8] Upon presentation to a health care provider, patients with probable dengue are assessed and classified into dengue with or without warning signs **(Figure I-1)**.[8] These classifications refer to the likelihood of an individual developing severe dengue, considering clinical observations made upon presentation.[8] Warning signs such as abdominal pain and thrombocytopenia are common in dengue cohorts, occurring in approximately 54% of hospital-admitted dengue patients according to one study, despite only 11% of all patients developing severe dengue.[9] Thus, the greatest utility of dengue classification

is to predict which patients are not at risk of severe disease and ease the burden of dengue on healthcare systems.

I-1.2.1 Clinical aspects of severe dengue

Severe dengue occurs in less than 1 in 100 symptomatic dengue patients and is potentially life threatening.[10] Treating consequences of hypovolemia by fluid replacement therapy is currently the only effective treatment in reducing morbidity, although, requires experienced supervision due to potential reperfusion injury.[9] When administered appropriately, fluid replacement therapy reduces the rate of mortality in severe dengue from 20% in untreated cases to 1%.[8, 11] Severe dengue symptoms begin to present as fever resolves and as viremia declines, due to increased immune activity.[12] This implies that a dysregulated immune response is causing disease, highlighting the importance of immune regulation during dengue. Discussed further in **Section I-2.8.4**, abundance of complement proteins and complement activation are correlated with dengue severity.

I-1.2.2 Mechanisms of dengue pathogenesis

The mechanisms of vascular leakage during dengue, which can lead to hypovolemic shock in extreme cases, are multifactorial.[13] Typically during infection, proinflammatory cytokines increase permeability of the vascular endothelium, which facilitates immune cells diapedesis.[14] However, the profile of cytokines released by immune cells during dengue, combined with dysregulation of endothelial architecture by DENV proteins, reduces the integrity of endothelial tight junctions.[13, 15, 16] The excessive production of proinflammatory cytokines, referred to as a ‘cytokine storm’ results from dysregulation of many immune mechanisms by DENV, discussed further in **Sections I-1.9-10** and **I-2.8**. It is currently hypothesised that, during dengue, the response of endothelial cells to excessive proinflammatory cytokines and secreted DENV proteins is the cause of endothelial hyperpermeability, resulting in vascular leakage. Thus, treatments that restore regulation of proinflammatory immune pathways during dengue are attractive targets for future therapies.

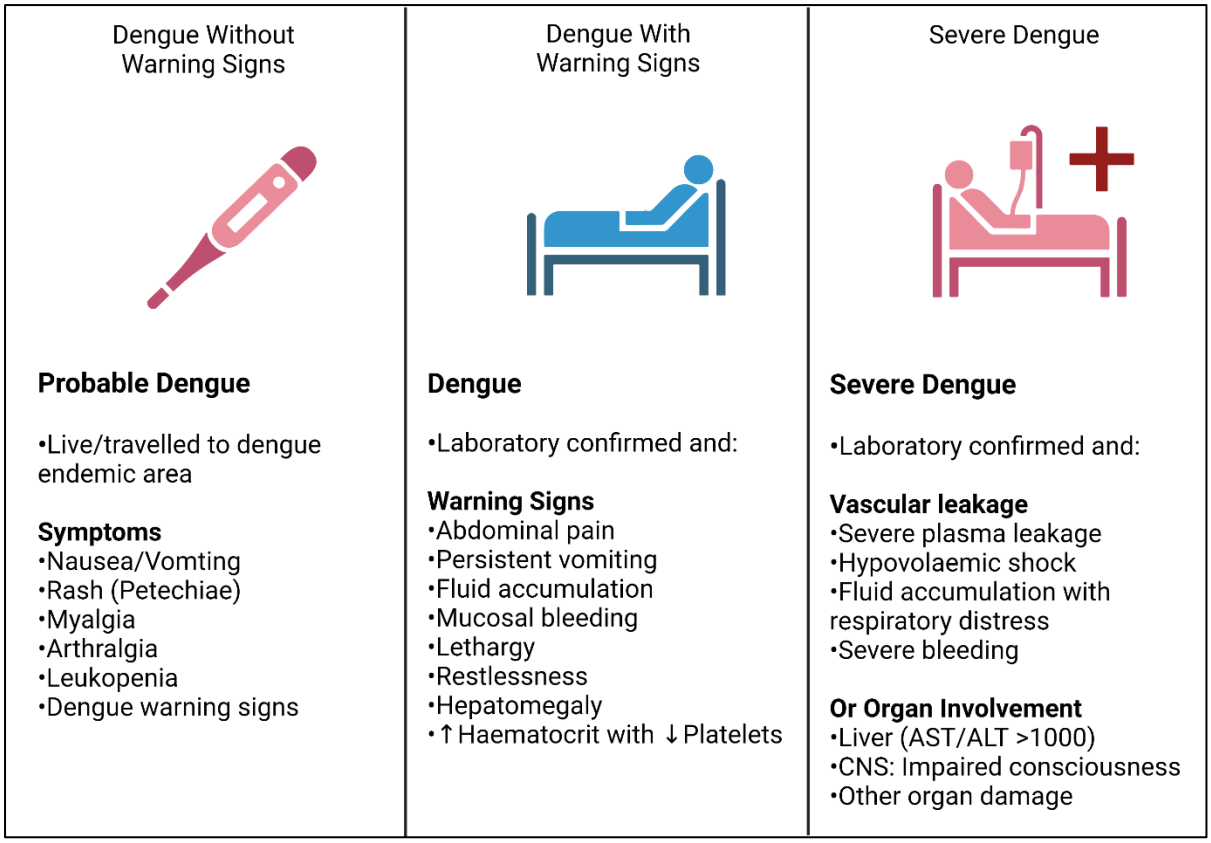

Figure I-1. Dengue with or without warning signs and severe dengue.

The WHO dengue classification system as described in 2009[8] for determining if a patient is displaying warning signs of severe dengue development. AST= aspartate aminotransferase ALT= alanine aminotransferase CNS = central nervous system.

I-1.3 Strategies to combat dengue

The efforts of researchers to reduce the burden of DENV spans across the fields of biochemistry to produce antivirals, immunology to produce vaccines and entomology to control the spread of mosquitoes and transmission of DENV.[17-19] Whilst vaccine development and mosquito control have been promising, antiviral development for DENV have been unsuccesful.[3] To date, no antiviral therapy has been licensed to treat dengue, although antivirals for other flaviviruses such as hepatitis C virus (HCV) have previously been trialled in dengue patients.[20, 21] Two examples of repurposed antivirals are celgosivir and balapiravir, however, neither drug effects dengue patient viremia, despite being well tolerated.[20, 21]

1.3.1 Development of vaccines to prevent severe dengue

There is no therapeutic goods administration (TGA), or food and drug administration (FDA) approved vaccine for DENV that can be used in seronegative individuals due to complex aspects of DENV virology and immunology. As a result of antibody dependent enhancement (ADE), the interaction between DENV and the immune system presents challenges. Due to ADE (described in **Section I-1.10.2**), imprudent vaccine design will cause more harm than good, increasing DENV infectivity and dengue severity in a naïve population. As such, vaccine development for DENV is a cautious process that has persisted with little success since the first attempts made by Professor Albert Sabin following World War II.[22, 23] After more than 80 years, the first DENV vaccine for commercial use, Dengvaxia, was made available by Sanofi Pasteur in 2016.[23] However, use in the 800,000 Filipino children without serotesting for anti-DENV IgG has resulted in controversy, litigation and removal of the vaccine from distribution in the Philippines, after it led to investigation of 100 severe dengue deaths linked to Dengvaxia.[24] Goals of future vaccines include tetravalent coverage, no evidence of ADE and safe use in seronegative individuals.[25] Excitingly, TAK-003 (Takeda) is a tetravalent dengue vaccine under licensure in select countries at the time of writing, following a successful 4.5 year, phase III clinical trial, demonstrating both efficacy and safety.[26]

1.3.2 Vector control to reduce the spread of DENV

Controlling mosquito populations by mosquito fogging and reducing man-made mosquito habitats, which sustain urban mosquito populations, is an effective method of controlling DENV transmission rates.[27] More sophisticated approaches to

controlling mosquito-human transmission of DENV involve transgenic mosquito populations and engineering endosymbiotic bacteria.[28] Wolbachia is an endosymbiotic bacterium that infects *A. aegypti* and astonishingly, suppresses DENV replication in infected mosquitoes.[29] In Australia, Wolbachia-infected mosquitoes were released in collaboration with the World Mosquito Program in Townsville, Queensland and infected a vast majority of the wild *A. Aegypti* population. This abolished mosquito-acquired DENV infections in Townsville by 2018.[30] These results are promising, not only for the control of DENV but other arboviruses transmitted by *A. aegypti*, such as Zika virus (ZIKV).

I-1.4 Models for studying DENV

Researching DENV *in vitro* and *in vivo* is crucial for elucidating the intricate mechanisms of infection and host response. Characterisation and definition of the response in cell lines and animal models following DENV-infection can provide insight into the mechanisms of infection and the immune system. However, there are some confounding factors inherent in each of these research models.

1.4.1 Models of DENV infection

DENV can infect many human cell lines *in vitro*, but it does so with varying levels of success.[31, 32] Disparities between infection rates and inherent issues with use of immortalised cell lines obfuscate the relationship between *in vitro* and *in vivo* infection. DENV typically infects monocytes, macrophages, dendritic cells and disrupts endothelial cell (EC) function *in vivo*. Thus, researchers, including in our laboratory, have investigated DENV infection using primary cells, such as monocyte derived macrophages (MDM) and human umbilical vein endothelial cells (HUVEC).[33, 34] The use of these primary cells provides the best insight into the context of DENV infection *in vitro.* However, primary cells are not ideal for experimentation due to polymorphic phenotypes, leading to heterotypic response to infection. Also, macrophages and HUVECs require ethics approval, greater expense of time and cost to use than immortalised cell lines. Thus, the first experimental aim of this thesis was to determine whether any cell line would recapitulate observations previously made in primary cells with respect to complement production and DENV infection.

There is currently no animal model for DENV infection that will induce the symptoms observed in humans without some caveat. There are some non-human primate (NHP) models which are permissive to DENV.[35] However, *in vitro*, viral replication is

decreased 1,000-fold in NHP tissue compared with human tissue.[36] NHP models are both costly and challenging to acquire and even if these challenges are overcome, DENV-infected NHPs do not demonstrate disease *in vivo*.[35, 36]

Mouse models of DENV infection also have limitations, as immunocompetent mice are resistant to DENV infection.[37] Ablating the interferon (IFN) response in mice, as in AG129 (Interferon (IFN) α/β/γ receptor deficient) mice, permits DENV infection with disease although, these models cannot be used to characterise the role of IFN during DENV infection.[38] One clear advantage in using AG129 mice is that they will develop vascular leakage symptoms as observed in humans.[38] Other mouse models, including intracranially-inoculated wild-type mice and human implant models (humanised mice), permit DENV infection but do not induce vascular leakage.[39, 40]

Other research groups have established different models for studying DENV in mice through mouse-adapted DENV strains. D2Y98P is a human isolate strain of DENV but is often utilised as it can reproduce human-like dengue symptoms in mouse models better than some mouse adapted strains.[41] Using D2Y98P Shresta *et al.* (2006)[42] instead developed a mouse adapted DENV strain through alternatively passaging human DENV between mosquito and mouse cells *in vitro*. Here, they were able to raise D2S10 which caused disseminated infection, which was not seen for D2Y98P-infection in the same study.

Previously, AG129 have been used by our laboratory to examine the effects of DENV infection on complement protein production. Cabezas *et al.* (2018) found that factor H (FH) and factor B (FB) mRNA concentrations were increased during infection but this did not influence concentrations of these proteins in the blood. This is disparate from the observations made in humans, discussed further in **Section I-2.8.4**. Further analysis into mouse and human promoter elements for these genes demonstrated that the IFN response is an important part of upregulation for complement proteins. Therefore, this thesis focussed on *in vitro* and *in silico* experimental methods which better represent interactions between DENV and complement *in lieu* of an IFN competent mouse model that demonstrates dengue disease.

I-1.5 The DENV host cycle

DENV infects both humans and *Aedes aegypti* mosquitoes in a cyclic manner. To the detriment of the mosquito, DENV infects the salivary glands and nervous system of *A.*

Aegypti.[45] Infecting these sites impacts mosquito behaviour, increasing time taken to feed and reducing the mosquitoes' fitness but improving viral delivery to humans.[45] In humans, DENV infection results in high titres of virus in the blood which improves the probability of infecting mosquitoes that feed on infected blood.[46]

I-1.6 DENV Virology

DENV is an enveloped, positive sense, single stranded RNA virus belonging to the *Flavivirus* genus of the Flaviviridae family.[47] DENV is closely related to other flaviviruses of clinical significance including ZIKV, West Nile Virus (WNV) and Japanese Encephalitis Virus (JEV).[47] Understanding the genome, structural and non-structural proteins of DENV help to define the mechanisms by which DENV, and sometimes other flaviviruses, infect, replicate, and cause disease in humans. Additionally, defining the aspects of viral structure and replication can reveal targets for future anti-viral drug development.

I-1.6.1 The DENV genome

The DENV genome is 10.7 kilobases (kb) long and encodes a single polyprotein which is cleaved by host and viral proteases into the 3 structural (capsid, membrane, and envelope) and 7 non-structural DENV proteins (Non-structural- 1, 2A, 2B, 3, 4A, 4B and 5) **(Figure I-2A)**. The utility of the DENV genome extends beyond translation of just the 10 proteins it encodes. For example, sub genomic flavivirus RNAs (sfRNA) are non-coding RNA elements important for maintaining genomic stability.[48-52]

I-1.6.2 The structural proteins of DENV

The DENV virion consists of the RNA genome concentrically surrounded by the capsid (C), which is surrounded by the membrane (M) and lipid envelope into which the viral envelope (E) protein is inserted **(Figure I-2B)**. These components assemble to form an immature virion that requires host-protein cleavage to form an infectious viral particle during exocytosis.[3]

The DENV capsid consists of 240 C protein copies to form an icosahedral structure approximately 12 kDa in size. Within the capsid, the DENV RNA is anchored to the internal capsid walls whilst the external capsid motifs bind to M-E structures.[31, 53] To form the virion, the DENV C subunits self-assemble on newly synthesised DENV RNA and bind each other by electrostatic attraction.[54] The promiscuity of C for all RNA is compensated for by the vesicle formation of DENV replication complexes in the

endoplasmic reticulum (ER) which reduces availability of host RNA to assembling virions.[55]

The M-E protein structure in an immature virion is composed of 180 PrM (18.8 kDa) and E (46 kDa) subunits that first form homodimers and then bind 1:1, PrM:E, to form 90 heterodimers.[56] Initial formation of the M-E structure produces immature virus and requires cleavage by the host protein, furin, to remove the Pr motif immediately prior to release.[31] Retention of the Pr motif until particle maturation prevents premature re-binding of DENV to the host cell.[57]

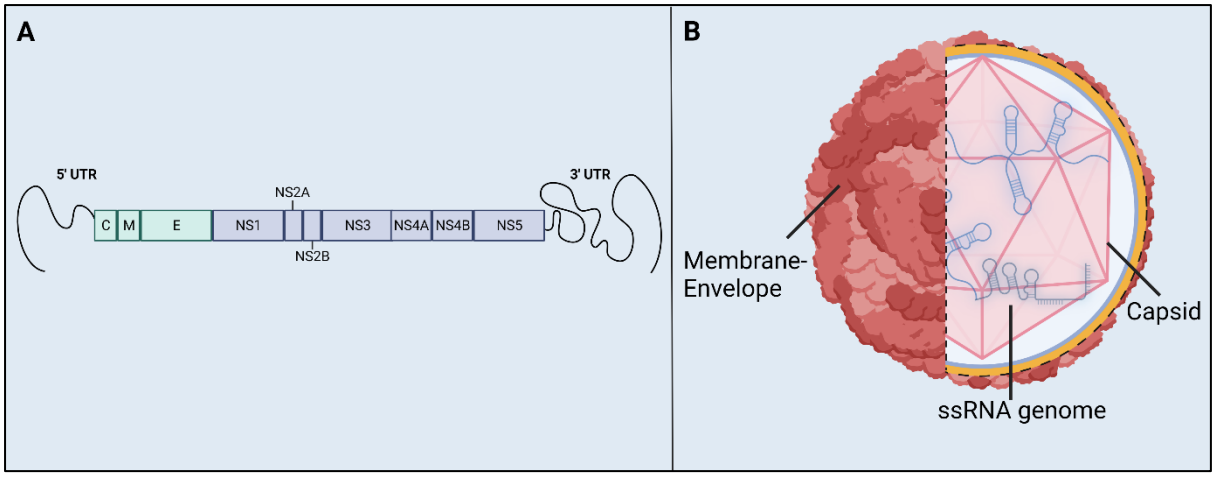

Figure I-2 The DENV genome and structure of an assembled virion.

(A) The DENV genome which encodes the DENV polyprotein that contains the capsid, premature membrane, envelope and NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 proteins. **(B)** The assembled and mature dengue virion displaying positive sense ssRNA within the icosahedral capsid structure, concentrically surrounded by membrane-envelope heterodimers. UTR = Untranslated Region, C = Capsid, M = Membrane, E = Envelope, NS = Non-structural proteins, ssRNA = single stranded RNA. Image A adapted from Alves *et al.,* (2021)[58]. Images created in BioRender.

I-1.7 DENV non-structural proteins

The DENV non-structural proteins (NS) are the seven proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) which have been observed to assist viral replication by directing host proteins, disrupting immune-response pathways and replicating the DENV genome.[3]

DENV-NS1 is secreted from infected cells, is highly abundant in patient blood during infection and is still detectable for 2-3 days after DENV RNA is no longer detectable in blood by qPCR.[59] Due to greater stability than DENV RNA and the high abundance of NS1 in patient blood, NS1 is one diagnostic marker used for point of care testing.[59, 60] The NS1 protein is multifaceted and has functions both intra- and extracellularly.[61] Within the cell, monomeric NS1 (52 kDa) binds DENV RNA, recruits ribosomal proteins to the ER and co-localises more than 30 other intracellular proteins to the cytoplasm.[62] Dimeric NS1 is translocated to the cell membrane.[61] Hexameric NS1 is the only secreted DENV non-structural protein and is directly associated with pathogenesis, specifically with regard to vascular leakage and inducing cleavage of glycosaminoglycans (GAGs) and sialic acid from the endothelial glycocalyx.[15, 63, 64] Also, DENV-NS1 induces inflammation, as it has been demonstrated to bind TLR-4 *in vitro*, eliciting cytokine production. Both secreted and membrane bound DENV-NS1 activate complement *in vitro* which also contributes to inflammation.[64-66]

NS3 and NS5 are the only enzymes of the DENV NS proteins. NS3 is a 70 kDa, bifunctional protein, that both unwinds the packaged DENV genome in preparation for replication and is a protease that post-translationally cleaves the DENV polyprotein assisted by the cofactor NS2B.[67] NS3 recognises and cleaves 16 different consensus sequences in the DENV polyprotein to produce individual DENV proteins.[57, 68] NS5 is the largest DENV protein (102 kDa) and is both the DENV RNA dependent RNA polymerase that replicates the genome and methyl transferase that methylates the DENV RNA cap as an essential post-replication modification.[69, 70] NS2A/B (22 kDa/14 kDa respectively) and NS4A/B (10 kDa/25 kDa respectively) are transmembrane anchor protein heterodimers that annex the virion within the ER for translation of the viral protein by sequestered ribosomes.[71] Additionally, DENV replication occurs in the ER and is enhanced by NS4A, which induces ER hyperproliferation, increasing sites for DENV replication. ER hyperproliferation also decreases the opportunity for

detection by cytosolic pattern recognition receptors (PRRs)[72] discussed further in **Section I-1.9.1**.

I-1.8 The DENV replication cycle

DENV replication is a multi-step process that begins with receptor-mediated endocytosis and concludes with cell-facilitated shuttling of the virus for exocytosis.[73] DENV hijacks, directs, exploits, and interferes with intracellular processes at the expense of the host cell's structure and resources in order to replicate. DENV is capable of infecting both humans and mosquitoes, however, by different receptors for each host. In mosquitoes, the prohibitin receptor is reportedly the primary facilitating receptor for DENV binding and entry.[74] In dendritic cells, the putative first cells to encounter the virus upon inoculation in the human.[75] DENV binds dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) for facilitated entry.[75] Fundamental to the phenomena of ADE is DENV uptake by fragment crystallisable γ receptor (FCγR) bearing cells discussed in **Section I-1.10.2**. There are many other candidate human cell surface molecules for DENV binding and entry such as CD14 and TIM/TAM receptors and GAGs.[74]

As the DENV genome is positive sense, single stranded RNA, it is directly available for translation, which is performed by host ribosomes, directing them to synthesise the DENV polyprotein.[76] Afterwards, proteolytic activation of the first DENV polyprotein into its subunits allows NS4A to induce ER hyperproliferation and recruitment of host ribosomes for further polyprotein translation and genome replication within vesicle packets. To replicate the DENV genome, NS5 first synthesises a complementary negative sense template strand, followed by semi-conservative replication to then produce thousands of genome copies per original template strand.[77] Following translation, the DENV polyprotein is cleaved by host proteases and then NS3, assisted by NS2B to produce the 10 distinct proteins.[78] The structural proteins assemble, as previously mentioned and the immature virion is transported out of the cell by hijacking the secretory pathway.[79] During Golgi transport, the outer structure of the virion rearranges to expose prM motifs available for furin cleavage.[79] Furin cleavage is the final stage of DENV virion maturation and upon exocytosis, the viral particle is infectious and the replication cycle repeats **(Figure I-3)**.[79]

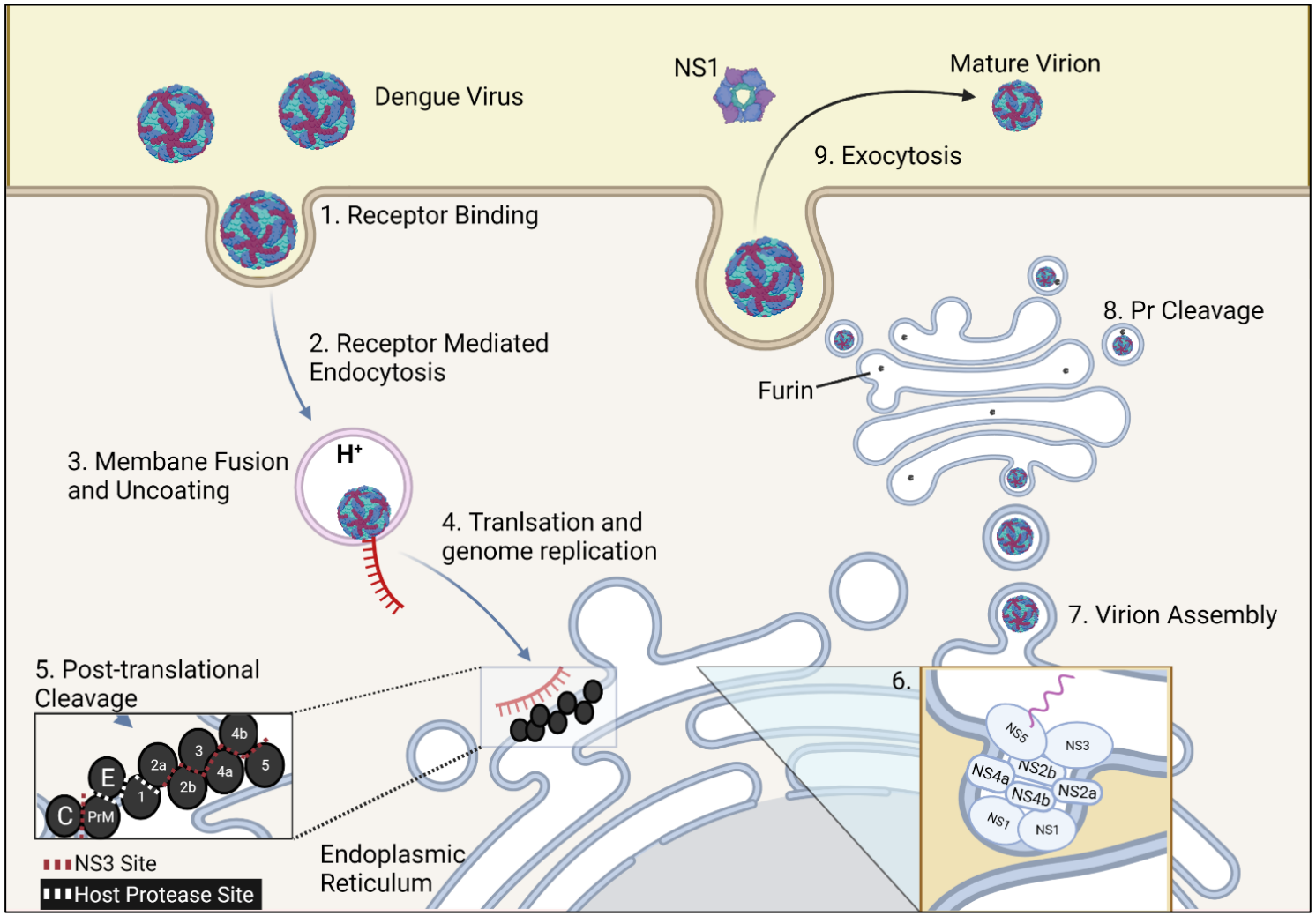

Figure I-3 The replication cycle of DENV.

(1+2) DENV enters cells via receptor-mediated endocytosis and **(3)** fuses to the endosomal membrane, uncoats in response to endosomal acidification and releases the RNA genome into the cytosol, **(4)** where it migrates to annexed vesicles in the ER for translation and genome replication. **(5)** The host ribosome translates the whole genome into a single polyprotein, which is cleaved into active proteins by host (red dashed line) and viral NS3 proteases (white dashed line). **(6)** Genome replication occurs in the vesicle packets DENV produces in the ER assisted by NS proteins **(7)** before it is packaged by the structural proteins and shuttled by the Golgi apparatus to the cell membrane. **(8)** The host protease furin cleaves the pr motif from prM to mature the virion **(9)** before it is exocytosed and is secreted. Image created in BioRender, based on (Uno and Ross, 2018)[79].

I-1.9 DENV and the innate immune system

The innate immune system is a powerful, broad set of mechanisms for defence against potential pathogens. Importantly, innate immunity is vital in controlling DENV replication before the adaptive immune system can act.

I-1.9.1 Host innate immune systems for detecting viral infection

Detection of the DENV ssRNA genome in the cytoplasm or endosome by PRRs triggers an intracellular immune cascade. Endosomal helicase toll-like receptor 3 (TLR-3), TLR-7 and TLR-8 as well as cytosolic helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation associated protein 5 (MDA5), bind and are activated by cytosolic and endocytosed dsRNA to induce expression of innate immune proteins. Additionally, mitochondria under stress during infection will release mtDNA and activate the DNA sensing protein, cyclic GMP-AMP synthase (cGAS) **(Figure I-4)**.[79] PRRs can effect a decrease in the ability of DENV to replicate. For example, *in vitro* RIG-I and MDA5 deficient Huh7 produce 10-fold more DENV RNA by 48 hours than Huh7 with RIG-I and MDA5.[80] In the same model of DENV-infected Huh7, TLR-3 knockout cells produced 2.5-fold higher DENV RNA by 48 hours post infection (hpi).[80]

PRRs do not directly reduce viral replication, instead, they activate cascades of intracellular proteins that induce antiviral genes.[79] During DENV infection, the PRRs described above are activated, which initiates downstream induction of innate-immune proteins.[79] In response to cGAS-DNA binding, cGAS will produce cyclic GMP-AMP (cGAMP), a STING agonist that promotes STING-mediated recruitment of TANK-Binding Kinase 1 (TBK1) and interferon regulatory factor-3 (IRF-3).[79, 81, 82] TBK-1 initiates autophosphorylation following STING binding and then activates IRF-3 and IRF-7.[83] Activated IRF-3 and IRF-7 will either homodimerize or heterodimerise, causing translocation to the nucleus. IRF-3 and IRF-7 then promote transcription of the type I interferons (IFNs), IFN-α and IFN-β.[84-86]

In a study investigating the interactions between, IRF-3, IRF-7 and DENV infection, transgenic mice were used to assess their relationship *in vitro*. Mice deficient in both IRF-3 and IRF-7 demonstrated 1,000-150,000-fold higher levels of DENV viral RNA than wild-type 24 hpi, produced lower levels of IFN-α and IFN-β and demonstrated delayed interferon stimulated gene (ISG) production.[87] Thus reinforcing that IFN-α and

IFN-β are highly potent antiviral signalling molecules against DENV, as observed *in vivo*.

Another transcription factor activated during DENV infection is Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB).[79] NF-κB is a potent inducer of cytokines during infection, and is located in the cytosol when inactive.[88] Here, NF-κB is anchored to IκBα which prevents NF-κB from migrating to the nucleus.[88] When activated by viral nucleic acid, PRRs such as TLR-3, TLR-7, RIG-I and MDA-5 activate downstream cascades that ultimately induces IκBα ubiquitination (degradation), freeing NF-κB to translocate to the nucleus.[89] NF-κβ activation induces important proinflammatory mediators produced during dengue that are correlated with worsened patient outcome, such as interleukin-6 (IL-6), IL-8 and tumour necrosis factor-α (TNF-α) **(Figure I-4)**.[79, 90]

The transcription factors activated in response to pathogen associated molecular pattern (PAMP) recognition will upregulate gene expression of anti-viral, proinflammatory cytokines such as IFNs mentioned above, ILs and TNF-α. Cytokines are typically small (~10-40 kDa), secreted signalling molecules of the immune system used for both autocrine and paracrine communication.[91] During infection, cytokines promote vascular permeability, leukocyte migration, lymphoid cell proliferation, and induction of genes with antiviral properties.[81, 92, 93]

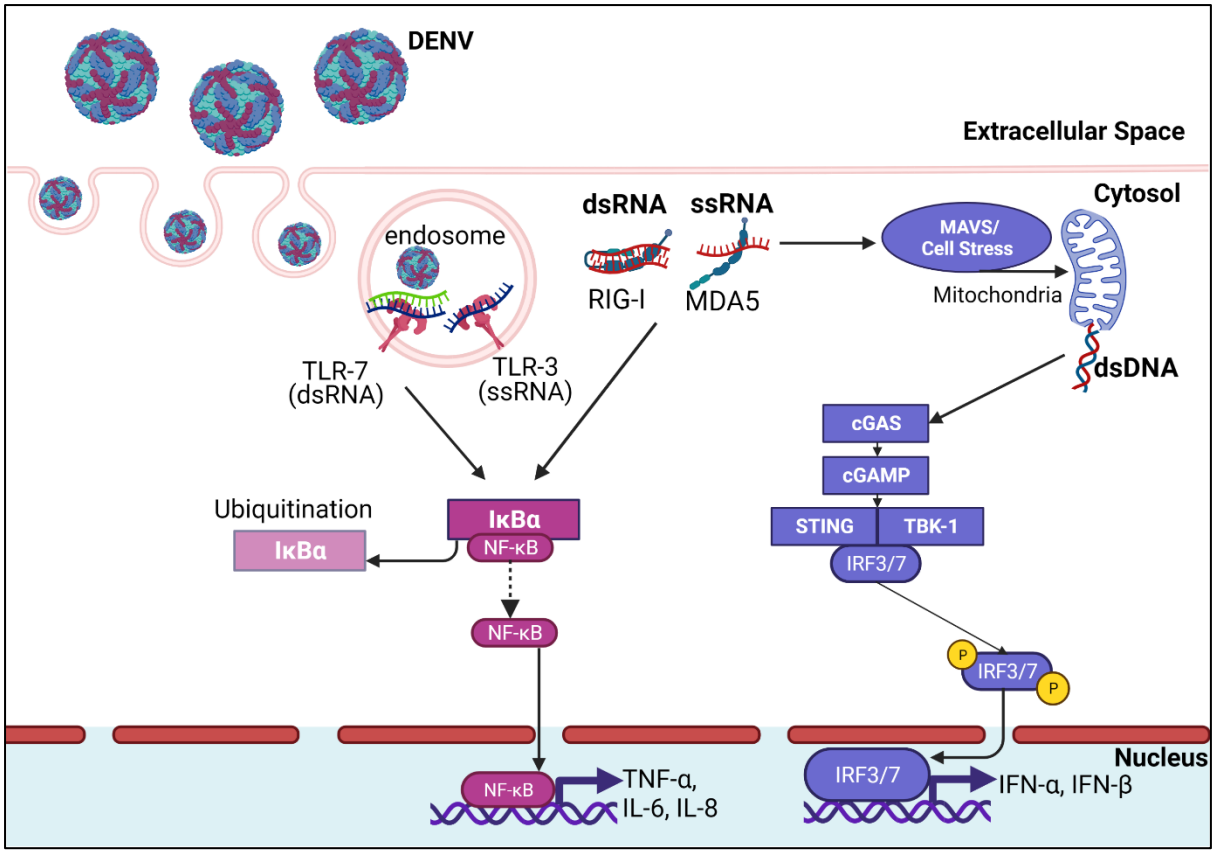

Figure I-4 DENV infection elicits host-antiviral responses activated by endosomal and cytosolic helicases.

DENV binds and infects permissive cells and is internalised by the cell, within the endosome. TLR-3 and TLR-7 are activated by detection of ssRNA and dsRNA respectively within the endosome. Receptor activation results in downstream degradation of the NF-κB anchoring protein, IκBα, through ubiquitination, allowing NF-κB to translocate the nucleus. MDA5 and RIG-I are also activated by ssRNA and dsRNA respectively also allowing NF-κB translocation. NF-κB induces transcription of proinflammatory cytokines such as TNF-α, IL-6 and IL-8. Additionally, RIG-I and MDA5 activation activates MAVS, inducing mitochondria to release dsRNA, this process can also be induced by cell stress caused by infecting DENV. Mitochondrial DNA release, activates cGAS which produces cGAMP, an agonist of STING. STING activation recruits TBK-1 which phosphorylates IRF-3 and IRF-7. Phosphorylated IRF-3 and IRF-7 translocate to the nucleus, enhancing transcription of type I IFNs, IFN-α and IFN-β. IFN = Interferon TRAF = Tumour Necrosis Receptor Associated Factor TLR-3 = Toll Like Receptor 3, RIG-I = Retinoic Acid Inducible Gene I, MDA5 = Melanoma Differentiation-Associate Protein 5 IRF= Interferon Regulatory Factor, DENV = Dengue Virus cGAS = cyclic GMP-AMP synthase, STING = Stimulator of IFN Genes. Image created in Bio Render, adapted from [79].

I-1.9.2 Cytokines produced during dengue affect patient outcome

TNF-α is an example of a cytokine elevated in dengue patient serum and is induced by DENV-infection *in vitro* but is directly correlated with increased dengue severity. Observations in patients have shown elevated TNF-α in severe dengue patients relative to those who did not develop severe dengue. This was observed by Kamaladasa et *al.,* (2016)[94] (n=25 vs 11, acute vs severe dengue) and Osuna-Ramos et *al.,* (2018)[95] (n=25 vs 16, acute vs severe dengue) but not Masood et *al.,*(2018)[96] (n=39 vs 4, acute vs severe dengue) which was potentially due to too few severe dengue patients recruited. Importantly, our laboratory has previously demonstrated that MDM produced increased TNF-α following DENV infection, however, inhibiting TNF-α in MDM did not affect the replicative ability of DENV.[97] Additionally, anti-TNF therapy has been shown to reduce mortality from 100% to 40% in a lethal mouse model of DENV infection.[98] Therefore, TNF-α is likely dispensable during DENV infection, yet is directly correlated to increased disease severity. As a result, TNF-α has previously been a therapeutic target for dengue although, without success.[97, 99]

Conversely, IFNs are especially important in inducing an antiviral state by enhancing expression of a plethora of proteins that defend against intracellular pathogens. *In vitro*, pre-treatment, of human foreskin fibroblasts, HepG2, K562, U-937, THP-1 monocyte leukemic cells, baby hamster kidney clone 21 cells (BHK-21) and MDM with IFN-α, IFN-β and IFN-γ up to 4 hours prior to infection reduces viral replication by up to 1,000-fold.[93] Additionally, wild type mice are not able to be infected by intravenous injection of most DENV strains as described in **Section I-1.4.2**. however, G129 mice, deficient in IFN-γ receptors (IFN-γR), A129 mice, deficient in IFN-α/β receptors (IFNAR) and AG129 mice, deficient in both IFNAR and IFN-γR, are permissive to DENV infection.[100] Johnson and Roehrig, (1999)[101] demonstrated that of these IFN deficient models (A129, G129 and AG129), AG129 were the fastest to succumb to infection, followed by G129 mice which is likely due to the combined deficiency of both type I and type II IFN response.

I-1.9.4 ISGs inhibit DENV replication

Activation of IFN α/β receptor (IFNAR) by detecting extracellular IFN initiates the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway.[79] JAK/STAT activation initiates a signalling cascade that results in IFN-stimulated response element (ISRE) activation and induction of hundreds of ISGs.[79] The STAT pathway is directly downstream of IFNAR receptor activation and is an

important early phase mechanism of host anti-DENV defence.[90] As IFN is an effector of innate immunity but does not directly inhibit viral replication, its effects can be suppressed by targeting the STAT pathway. Ashour *et al.,* (2010)[102], demonstrated that, after DENV infection, wild-type mice are able to reduce lymph and spleen DENV RNA and infectious virus to below the detectable limit of qPCR and plaque assay respectively within 18 hours. Conversely STAT2$^{-/-}$ mice still had detectable DENV RNA and infectious virus 32 hpi for the lymph and spleen, although DENV RNA was no longer detected by 60 hpi. The results of the DENV-infected STAT2$^{-/-}$ mice therefore demonstrated the importance of STAT and the IFN response in acute antiviral defence against DENV. In the same study, the authors demonstrated that the ability of murine cells to inhibit DENV replication *in vitro* was attributable to DENV-NS5-STAT2 binding, whereby human STAT2 but not mouse STAT2 was targeted for degradation by DENV-NS5. This is likely due to the importance of STAT2-mediated induction of ISGs, which help sustain an antiviral response during infection. In the case of DENV specifically, IFITM2, IFITM3, viperin, ISG20 and double-stranded-RNA-activated protein kinase are ISGs that strongly inhibit DENV replication processes by interfering with genome replication and translation of nascent viral proteins.[103] Other genes important for protecting the host from immune-mediated damage during dengue are also upregulated in response to cytokines such as those that regulate complement.[43, 44] In some instances, as in the complement system, the ability of the patient to prevent autoreactivity of immune pathway during infection can be a determinant of disease severity, which is discussed in greater detail in **Section I-2.8.4**.

I-1.9.5 Mechanisms of evading innate immune pathways by DENV

Every component of the DENV genome, coding, and non-coding, has evolved to efficiently facilitate replication of DENV in both mosquito and human cells. One facet of ensuring successful viral replication is subversion of the innate immune response which threatens to inhibit or terminate the process.[104]

Together, the 10 DENV proteins are capable of downregulating IFNAR signalling pathways[90], reducing type I IFN production[105], destroying STING complex subunits[106], inhibiting RIG-I-dsRNA binding, degrading cGAS[82], dysregulating mitochondrial antiviral defence[82], degrading STAT2[107] and preventing phosphorylation of TBK-1[83], IRF3[83] and STAT1[108]. The extent to which DENV prevents innate immune regulation demonstrates the complexity of these pathways and the importance of these genes in

preventing viral replication. For example, in the case of IFNs, DENV NS2B/3 protease complex interferes with nascent IFN-α/β mRNA production by cleaving the innate mediatory protein complex STING.[90] The STING complex is a key mediator of cytokine production by causing downstream activation of NF-κβ to promote production of the interleukins as described in **Section I-1.9**.[109] To further dampen the effects of IFN mediated innate immunity, DENV NS2A, NS4A and NS4B block STAT1 and STAT2 phosphorylation, preventing activation and induction of ISGs, thus attenuating the effect of IFNs further.[110]

I-1.10 DENV and the adaptive immune system

Although innate immunity is vital for moderating the ability of DENV to replicate during infection, adaptive immunity is important for removing DENV infected cells.[90] During secondary infections by a homotypic serotype, the adaptive immune system can also prevent disease.[12, 111, 112] Cells of the adaptive immune system can contribute to better patient outcome, although are not essential for eliminating DENV infection. For example, increased markers of activated CD4+ T-cell have been observed in children with subclinical dengue relative to symptomatic children following secondary infection.[113] Additionally, CD4+ T-cells collected from healthy donors, who had previously experienced DENV infection, were capable of eliciting cytolytic activity in DENV-infected cells *ex vivo*, reducing total viral load.[114] However, in IFN-deficient mice with depleted CD4+ T-cell populations, DENV viral titres are not significantly different from mice with normal CD4+ T-cell populations.[37] Although, IFN deficient models are poor representatives of CD4+ T-cell responses, as IFN is important for CD4+ T-cell differentiation and polarisation.[115] Regardless, the above data demonstrate that CD4+ T-cells are an example of a cell that protects from dengue symptoms and is beneficial during infection but is not vital.

I-1.10.1 Antibodies

Antibodies are an important component of the adaptive immune response to infection. Antibodies are Y-shaped secreted glycoproteins produced by B-cells as five isotypes, IgA, IgE, IgM, IgD and IgG where each isotype demonstrates unique patterns of localisation, antigen affinity and complement binding.[116] IgA, IgE and IgD will not be described further in this introduction. Antibodies produced by each B-cell are unique due to somatic recombination of the heavy and light chain genes during B-cell development.[117]

IgM and IgG are serum acting antibodies that exist as pentamers and monomers, respectively.[116] An antibody consists of an Fc region and 2 identical copies of a heavy and light chain called the Fab region.[117] Following antigen binding to the Fab region, the invariable FC region can bind cognate receptors of FC-receptor bearing cells. FC-receptor binding promotes antibody-dependent cellular cytotoxicity, phagocytosis or the classical pathway (CP) of the complement cascade through C1q, described in greater detail in **Section I-2.1**.[118, 119] Antibody binding of DENV can either neutralise infection or be 'non-neutralising' dependent on the number of bound antibodies and binding region.[103] The memory produced by the adaptive immune system is initially cross-protective, for heterotypic infections for a brief period of 2-3 months.[120] Following this window of protection however, other infecting DENV serotypes can benefit from circulating cross-reactive, non-neutralising antibodies enhancing infection, as described further in **Section I-1.10.2**.[12, 121]

I-1.10.2 Antibody Dependent Enhancement of Infection

ADE is the enhancement of infection by non-neutralising antibody binding of the DENV virion, which facilitates infectious viral uptake by FCγR-bearing immune cells such as monocytes and macrophages.[103] ADE is also known to enhance infection of influenza virus[122], HIV[123], SARS-CoV-1[124] and coxsackievirus[125] and is one of the proposed mechanisms responsible for increased disease severity after secondary DENV infection by a heterotypic serotype.[126] The most accessible DENV protein for external binding by antibodies is E. Previous studies have demonstrated that antibodies may bind E with high avidity and neutralise the virion or, conversely, fail to neutralise the virion and enhance heterotypic infection depending on the epitope **(Figure I-5)**.[127] Antibodies targeting conserved elements of the DENV envelope, such as the region of protein dimerisation, provide protection against both homotypic and heterotypic DENV reinfection and are therefore attractive targets for vaccine design. Whereas antibodies specific to the envelope fusion loop are cross reactive, non-neutralising, and proposed to enhance DENV replication by promoting FCγR-mediated endocytosis of infectious virus in macrophages.[128] DENV replication can also benefit from low titres of neutralising antibodies. In this instance, antibodies that bind DENV neutralise domains of the virion but not the entire particle.[129] This has been demonstrated *in vitro*, where DENV incubated with serially diluted antibodies will cause greater viral replication and infection in mature dendritic cells, monocytes and the U937 cell line.[130] This mechanism also has implications for disease severity in infants, as maternal IgG

from shared blood *in utero* and breast milk decreases after birth.[131] Therefore, at non-neutralising titres of DENV antibodies, infants are at higher risk of severe dengue via ADE than infants without circulating maternal IgG. Clinically, this translates to low incidence of severe dengue in infants from dengue endemic countries until 6 months of age, where rates of severe disease and infant mortality sharply increase.[129, 131]

To the detriment of the host, DENV ADE will also change the cytokine release profile of infected cells. For example, one experiment investigating ADE in the histiocytic lymphoma cell line U937, which demonstrates promonocytic properties, found increased proinflammatory cytokine induction during ADE of DENV. Here, proinflammatory cytokines IL-6, TNF-α and IL-12 p70 were higher than in cells directly infected with DENV **(Figure I-5)**.[130] As mentioned in **Section I.1.9.1**, immunopathogenesis during DENV infection is correlated with IL-6 and TNF-α concentrations, as well as other cyotkines.[15] Thus, ADE can dysregulate the response to viral uptake, increasing proinflammatory cytokines, worsening disease by promoting greater vascular leakage and decreasing immune cell-mediated viral clearance.

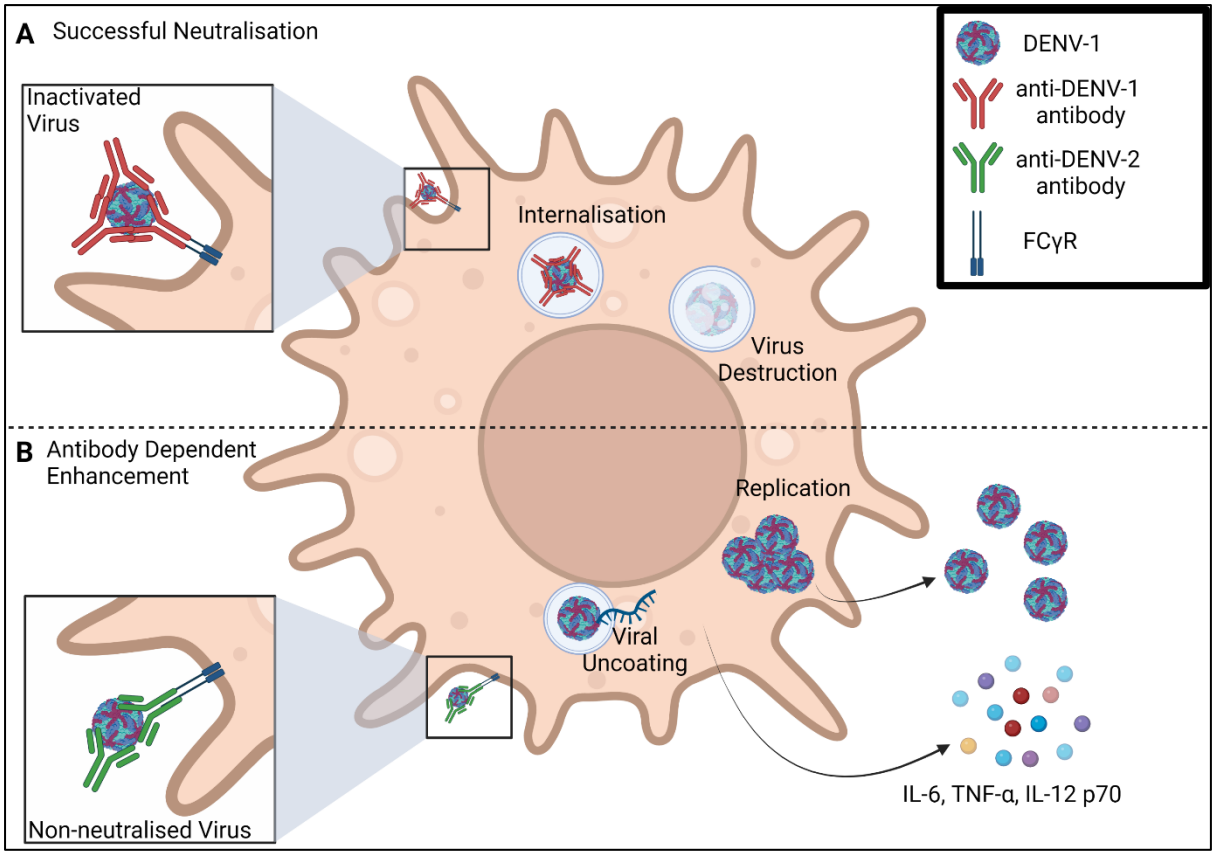

Figure I-5 Heterotypic infection with DENV enhances infection in FCγR-bearing cells.

DENV virions challenged with antibodies will be **(A)** neutralised during homotypic infection upon encountering antibodies for that serotype and destroyed by the FCγR-bearing cell following phagocytosis. **(B)** Virions heterotypic to the primary infection may be bound but not neutralised by circulating antibodies and will successfully infect the FCγR-bearing cells, infecting the cell and successfully replicating. ADE also causes increased release of proinflammatory cytokines IL-6, TNF-α and IL-12 p70. FCγR = Fragment Crystallisable γ Receptor. ADE = antibody dependent enhancement. DENV = Dengue virus. DENV-1/2 were used for the purpose of example and ADE can occur for all serotypes. Image created in BioRender.

I-2 The complement system

The complement system is a branch of innate humoral immunity, linking to and enhancing the function of adaptive immunity. The complement system is specific to vertebrates, although an analogous protein found in invertebrates suggests that complement has evolved from a prototypic immune protein 700 million years ago.[132]

There are more than 50 soluble and membrane-bound proteins that compose the complement system.[133] Primarily, complement facilitates the destruction and removal of pathogens, apoptotic cells and necrotic tissue.[133] Activating the complement system initiates a cascade of coordinated binding and proteolytic activation by complement proteins. Ordered, successive binding of complement proteins to a surface produces a scaffold for formation of the membrane attack complex (MAC) which fenestrate the membrane and initiates non-inflammatory apoptosis and cell debris clearance mechanisms.[134] Activation of this terminal pathway and complex formation can occur by three distinct pathways, these are the CP, mannose-binding lectin (MBL) and alternative pathway (AP) **(Figure I-6)**.[135]

I-2.1 The classical pathway of complement activation

The CP is probably the most well studied of the three pathways and is dependent on antigen-antibody binding.[136] In fact, the term complement is derived from the ability of the proteins to 'complement' antibody mediated clearance of antigens.[137] The C1 esterase complex typically initiates the CP by binding the FC region of IgM and IgG.[138] However, C1 has also been demonstrated to activate the CP by binding pathogen surfaces, C-reactive Protein (CRP), lipopolysaccharide (LPS) and some viral proteins.[138] Binding these molecules will initiate the complement cascade and opsonise the bound surface.[138] C1 complexes recruited to antigen-bound antibodies causes activation of a protease cascade. Here, C1 cleaves the circulating zymogen, C4 into C4a and C4b, C2 then binds C4b and is cleaved by C1s, which ultimately results in the formation of the classical pathway C3-convertase (C4bC2a). C3-convertases cleave C3 to produce the two active subunits, C3a and C3b, the latter being the larger surface binding protein common to all three pathways. C3b can then bind the C3-convertase to form the CP C5-convertase (C4bC2a3b). The surface-bound C5-convertase cleaves and activates circulating C5 into C5a and C5b. C5b then recruits C6, C7, C8 and C9 (C6-C9), which forms the membrane attack complex, a pore forming structure that induces osmotic cell death **(Figure I-6)**.[139] [134]

I-2.2 The mannose-binding lectin pathway of complement activation

MBL binds microbial PAMPs on bacteria, fungi and some viruses directly to initiate complement activation.[140, 141] MBL associated serine proteases (MASP1 and MASP2) with MBL from the lectin pathway cleave C4 into the active subunits C4a and C4b and C2 into C2a and C2b, producing the same C3-convertase that results from CP activation, at which point the two complement pathways converge **(Figure I-6)**. C3b produced by C3-convertases of the CP and MBL pathways can also act as a foundation for initiating the AP pathway.[142]

I-2.3 The alternative pathway of complement activation

The AP is constitutively active but specifically regulated on surfaces of the host and not pathogens.[143] Properly regulated AP activity is associated with strong, fast acting, and selective immunity.[144, 145] Conversely, poorly regulated AP activity is the cause of serious medical conditions that act on multiple organs, most prominently the kidneys.[146]

C3 is the inactive parent molecule of C3b and the most abundant complement protein in circulation.[147] Large quantities of C3 may be rapidly cleaved by the C3-convertases of either the CP or MBL.[145] C3 is inactive in circulation, however, low amounts of fluid phase C3 cleavage occurs spontaneously in a process called ‘tick-over’ as reviewed by Lachmann (2018)[148] who also first proposed the theory in 1974. Fluid phase C3b, formed by tick-over or the CP and MBL C3-convertases then initiate the AP.[148, 149]

Although C3b is constitutively produced, fluid-phase C3b is highly labile as it is inactivated by H_2O and therefore, has nanoseconds to bind an available surface, including those of host cells.[150] Surface bound C3b will then recruit FB binding, resulting in cleavage by Factor D to produce (FBb) and form a C3bBb complex, the AP ‘C3-convertase’.[135, 148] This convertase is then either stabilised by properdin or decays.[148] As C3b is both a subunit and product of the C3-convertase, formation of the AP C3-convertase results in a positive feedback mechanism.[135, 148] Decay accelerating factor (DAF), complement receptor 1 (CR1) and FH can all catalyse C3-convertase decay on host cells, narrowing the window of opportunity for further complement activation, which is important for inhibiting feedback mechanisms of the AP.[151, 152]

The AP C3-convertase is also the scaffold of the AP C5-convertase. Here, binding of yet another C3b molecule to the AP C3-convertase allows the complex to perform C5-cleavage i.e. a C5-convertase complex and the three pathways of complement activation converge after C5-convertase formation **(Figure I-6)**.[153]

I-2.4 The anaphylatoxins

The term anaphylatoxin refers to the ability of C3a and C5a to degranulate mast cells.[154] Anaphylatoxins, specifically C3a and C5a, are small complement protein cleavage products of C3 and C5 respectively **(Figure I-6)**, with potent cytokine action.[154-156] C4a is an anaphylatoxin by convention of nomenclature but does not produce effects akin to C3a or C5a and will not be discussed further.[155, 156] C3a and C5a are products of complement activation by all three pathways, they are responsible for increasing vascular permeability, recruiting immune cells, and inducing inflammation. However, anaphylatoxins do not induce inflammation directly, these are products of anaphylatoxin-mediated activation of platelets and mast cells which initiate degranulation, releasing proinflammatory cytokines.[156] Aberrant anaphylatoxin production can cause disease in the absence of infection, which occurs for example in allergic asthma.[157] Excessive anaphylatoxin production in response to overwhelming microbial infection during sepsis, can lead to hypotension and septic shock.[158]

I-2.5 The regulators of complement activation

The regulators of complement activation (RCA) are localised on chromosome 1q32 and are all composed of complement control protein domains (CCP) which are short consensus repeats (SCRs) of 60 amino acids. This is except for the VSIG4 receptor (also known as the CRIg receptor) that is composed of immunoglobulin domains. These proteins each control complement activation or MAC formation on healthy host-cell surfaces. FH contains 20 SCRs and is the most significant regulator of the AP, regulating AP activation at multiple stages.[136]

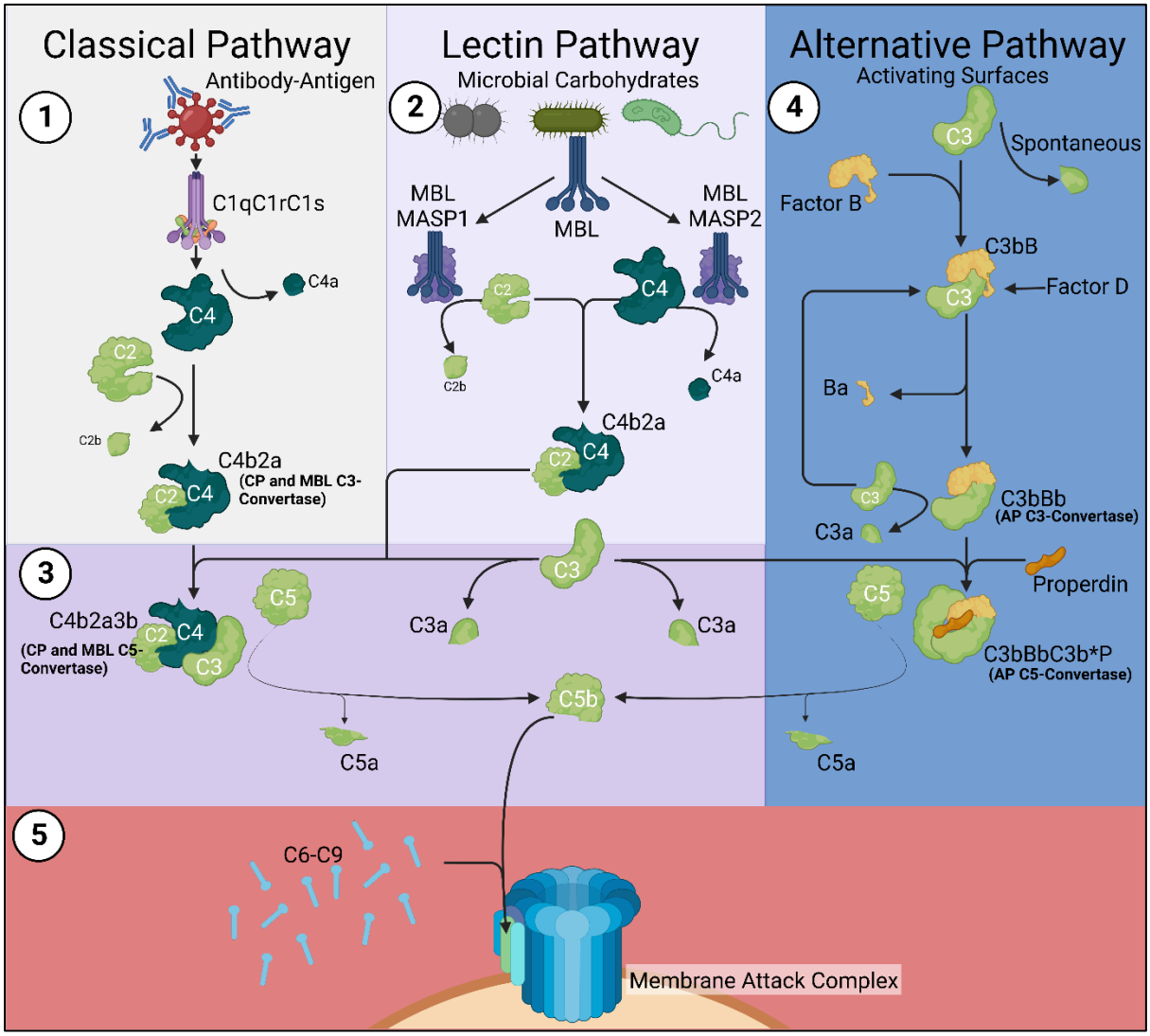

Figure I-6 The three complement pathways are activated in distinctly different manners.

(1) The classical pathway is activated by complement C1 complex (C1qC1rC1s) binding antigen-bound antibodies which can cleave C4 and C2 into C4a, C4b, C2a and C2b which form a C3-convertase. **(2)** The lectin pathway is initiated by multimers of mannose-binding lectin and MASP-1 and MASP-2, that also proteolytically cleave C4 and C2 to form a C3-convertase **(3)** C3-convertase sequesters C3b to form the C5-convertase for the classical and lectin pathway that cleaves C5 into C5a and the C5b active subunit required to initiate membrane attack complex formation. **(4)** The alternative pathway is activated by spontaneously formed, surface bound C3b, which binds factor B. Factor B is cleaved by factor D to generate Bb and Ba factor B subunits. C3bBb acts as the alternative pathway C3-convertase, which is part of a positive feedback loop, cleaving C3 into C3b for further C3-convertase formation. The AP C3-convertase forms the alternative pathway C5-convertase (C3bBbC3b*P) by acquiring one more C3b molecule and stabilisation by properdin. **(5)** C5b deposits on the cell membrane to recruit C6-C9 to form the membrane attack complex on an activating surface.

I-2.6 Complement Factor H

FH prevents AP feedback mechanism overactivity during the early stages of activation.[159] FH can specifically protect host cells from complement activation by binding host-specific ligands and prevents C3b deposition as well as FB-C3b binding. Host cell surfaces where C3-convertase have already formed can direct FH as a cofactor for the protease Factor I (FI) to irreversibly cleave C3b and inactivate C3-convertases **(Figure I-7)**.[160] Production of FH mainly occurs in the liver, as with most complement proteins.[161, 162] However, expression of FH by some cell types, including immune cells, heavily influences local AP activity.[163]

I-2.6.1 Structure and functions of the FH SCRs

FH is a 155 kDa protein, composed of twenty SCR domains and although similar, each region has different affinities for ligands such as C3b and host-surface proteins and proteoglycans.[164-167] The most significant regions with roles of AP regulation are FH_{SCR1-4}, FH_{SCR6-7}, and $FH_{SCR19-20}$ **(Figure I-8)**.

FH_{SCR1-4} is at the FH N-terminal and is a C3b binding domain which competitively binds the FB binding site of C3b, preventing C3b binding to FB and subsequent C3-convertase formation. It has also been shown that FH_{SCR1-4} has decay-accelerating activity of C3-convertase through cofactor activity for FI.[168] FH_{SCR1-4} is also important for dissociating already formed C3-convertases on non-activator (typically host cell) surfaces. This domain is not unique to the full-length FH protein but is also translated in the alternatively spliced isoform complement FH Like-1 (FHL-1) **(Figure I-8)**.[169]

FH_{SCR6-7}, like FH_{SCR1-4}, is not unique to full length FH but is important for host-cell recognition by binding heparan sulphate (HS) on host-cell surfaces. HS represents highly sulphated GAGs on cell surfaces and is thought to be a motif by which FH binds and protects the glycocalyx surrounding ECs.[170] FH_{SCR6-7} is a well-studied region of the FH molecule, as a SNP (rs1061170) in the HS binding site is strongly associated with increased risk (5.8-fold) of developing age-related macular degeneration (AMD).[171, 172] FH_{SCR6-7} is also the domain responsible for cofactor activity to assist FI-mediate cleavage of fluid phase C3b.

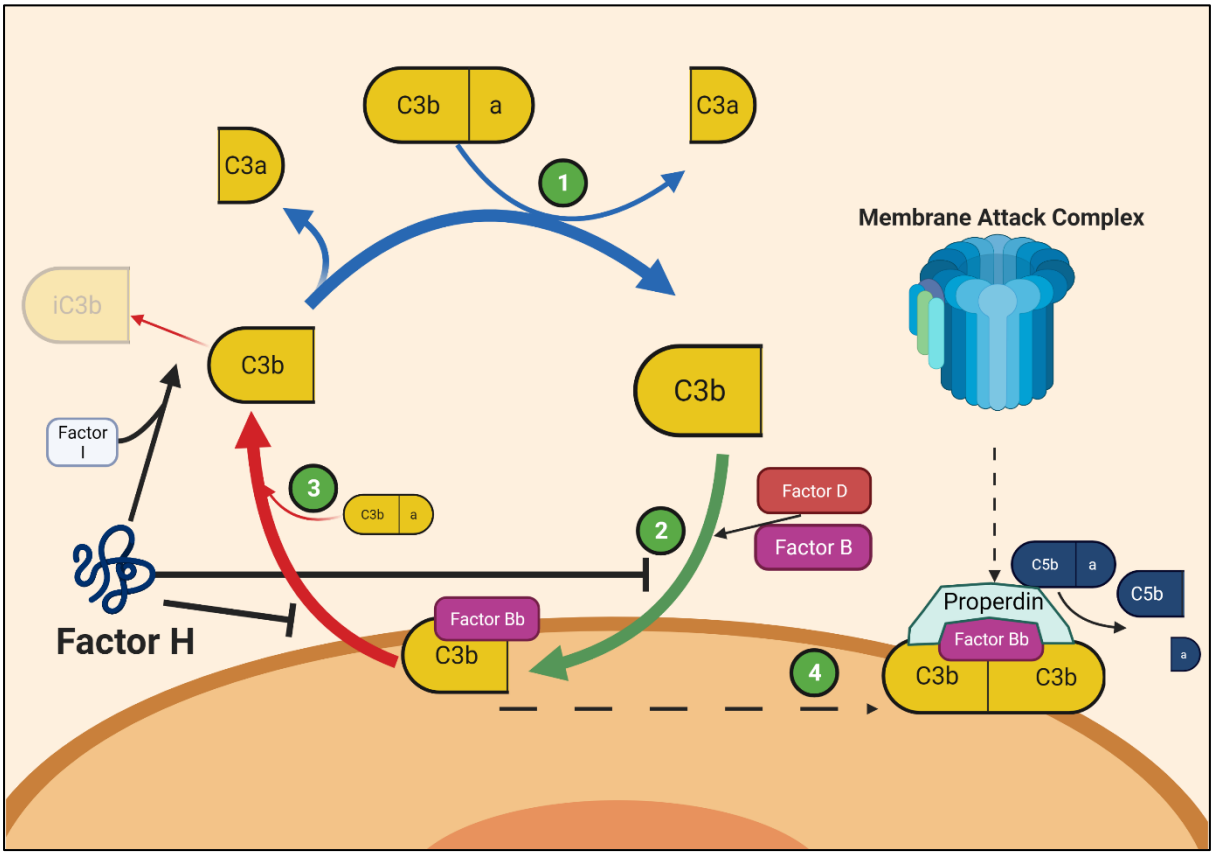

Figure I-7 Factor H regulates the alternative pathway at three stages of AP activation and feedback.

C3b is spontaneously cleaved at a low rate or by existing C3-convertases **(1)** which initiates alternative pathway by C3b depositing on cell surfaces and binding factor B. Factor B is cleaved by factor D to form the C3-convertase (C3bBb) **(2)** which amplifies C3 cleavage **(3)** and is the scaffold for the C5 convertase and promotes formation of the membrane attack complex **(4)**. Factor H can destabilise the C3-convertase, prevent C3bB from forming C3bBb (at step 2) and act as a cofactor for Factor I (at step 3) to inactivate C3b by proteolytic cleavage. Image created in BioRender.

$FH_{SCR19-20}$ is the C-terminal domain of the FH molecule and one of the most important domains for host cell binding, using HS and sialic acid as host-cell recognition motifs **(Figure I-8)**.[173] Additionally, $FH_{SCR19-20}$ has been demonstrated to be essential for FH-C3b binding. The current model for FH-C3b binding suggests that $FH_{SCR19-20}$ concomitantly binds cell surface polyanions such as sialic acid and C3b. $FH_{SCR19-20}$ has higher affinity for C3b than FH_{SCR1-4} for C3b. Here, $FH_{SCR19-20}$ has been demonstrated to have 1.6-fold greater C3b binding to immobilised C3b than FH_{SCR1-4} *in vitro*.[174] Due to the importance of C3b and sialic acid binding for regulation of the AP, the C-terminal domain of FH is essential to complement regulation. Hence, disruption of the C-terminus can ablate FH regulatory abilities, which has been observed *in vitro* and *in vivo* and is described in greater detail in **Section I-2.6.3**.

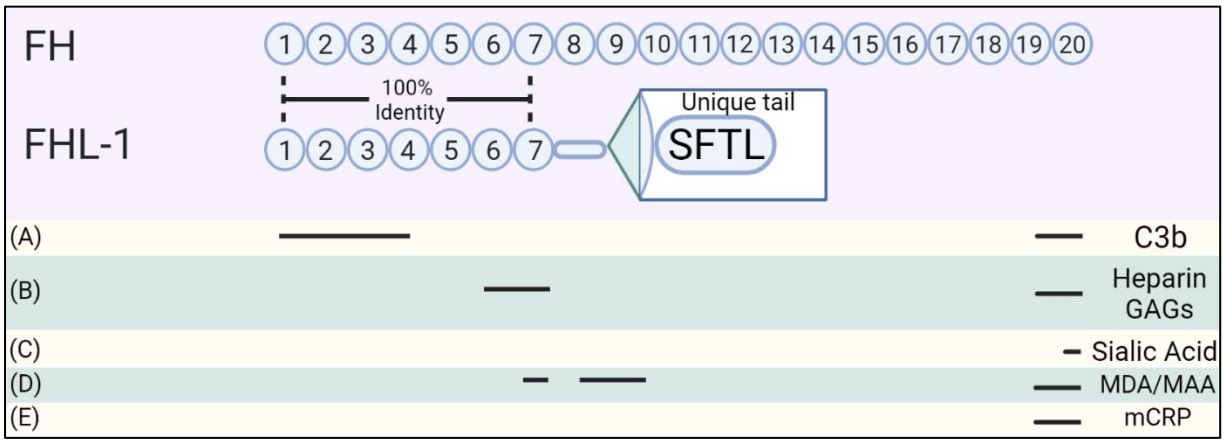

Figure I-8 Binding domains of FH and the alternatively spliced FHL-1 protein.

The SCRs of FH and FHL-1 are identical from SCR1-7 but diverge after SCR7 where FHL-1 has a unique four amino acid tail (Serine-Phenylalanine-Threonine-Leucine) and is then truncated. **(A)** C3b binds SCR1-4 on both FH and FHL-1 and $FH_{SCR19-20}$. **(B)** SCR6-7 on both FH and FHL-1 bind heparin and glycosaminoglycans, as does $FH_{SCR19-20}$. **(C)** Sialic acid binds FH_{SCR20}. **(D)** MDA and MAA bind SCR7 on FH and FHL-1 and $FH_{SCR9-10}$ and $FH_{SCR19-20.}$ **(E)** FH_{SCR20} binds mCRP. FHL-1=Factor-H-Like 1, GAGs = Glycosaminoglycans, MDA/MAA = Malondialdehyde/acetaldehyde adduct, mCRP = monomeric C-Reactive Protein. Image created in BioRender adapted from.[175]

I-2.6.2 Regulation and induction of FH expression

In silico analysis of the *CFH* promoter has predicted transcription factor (TF) binding elements canonically activated in response to infection, including an ISRE element and binding sites for NF-κβ and members of the STAT (STAT5 and 6) and IRF (IRF1, 4 and 7) families **(Figure I-9)**.[176] *In silico* analysis by Ormsby (2004)[177] also predicted transcription factor binding sites such as CCAAT enhancer binding protein, nuclear factor of activated T cells, cAMP-responsive binding element protein and glucocorticoid responsive elements **(Figure I-9)**. The presence of the above elements predicts responsiveness of the *CFH* promoter to transcription factors stimulated following stimulation of PRR described in **Section I-1.9**. The *CFH* promoter is also clearly under control of some liver-specific transcription factors driving homeostatic hepatic FH production although, these were not predicted with the techniques used by Cabezas, (2018) or Ormsby (2004).[145] No other attempt to define human hepatic *CFH* promoter elements have been published. *In silico* analysis of the murine *CFH* promoter also failed to identify sites for Hepatic nuclear factor1-4 (HNF) binding hypothesised by the authors.[178] The *CFH* promoter has not been experimentally characterised systematically; whereby newfound understanding of the *CFH* promoter is sporadically informed by empirical observations and their associations with disease.[179-183] Understanding of *CFH* gene induction would be greatly benefited by a thorough and consolidated approach of promoter element definition, as it appears FH expression is dynamic, especially during inflammation and infection.

Circulating FH concentrations are highly variable in blood (116-562 μg/mL) with no apparent effect on immunity between high and low concentrations and thus, probably exceeds the minimum concentration for controlling basal plasma complement activation in most individuals.[184] Even so, hepatic FH production cannot reach all sites of AP activation, which is evidenced by examples of extrahepatic homeostatic and inducible FH production as in the brain and monocytes respectively.[185]

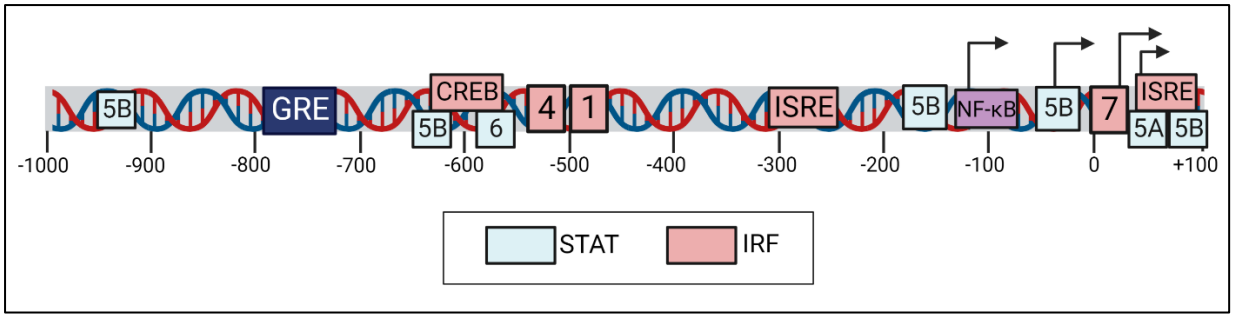

Figure I-9. *CFH* is predicted to contain binding elements for NF-κB and members of the STAT and IRF family.

The -1000bp to +100bp region of the FH promoter was analysed by the *in silico* promoter analysis tool MATINSPECTOR. Transcription factor binding elements and their location is pictured. Arrows represent predicted transcription start sites. NF-κB = Nuclear Factor kappa-light-chain-enhancer of activated B cells, STAT = Signal transducer and activator of transcription, IRF = Interferon regulatory factors, ISRE = Interferon stimulated response element, GRE = Glucocorticoid responsive element, CREB = cAMP response element-binding protein.

I-2.6.3 FH and disease

FH function is vital for preventing inappropriate activation of AP, both in fluid phase and on host surfaces. Thus, changes in FH concentration or molecules that interfere with FH's ability to act on host cell surfaces have the potential to cause disease.

I-2.6.4 FH prevents fatal AP self-activation through the C-terminus

The FH C-terminal is the most conserved region of FH in humans.[186] This is likely due to the devastating consequences of point mutations in the hydrophobic pocket of the C-terminus. As such, over 100 SNPs in the C-terminal region of the FH molecule are associated with serious complementopathy, causing atypical Haemolytic Uraemic Syndrome (aHUS).[187] In aHUS, aberrant complement activation causes thrombocytopenia and acute kidney failure and, if left untreated, has high rates of mortality (25%).[188] Autoantibodies for the C-terminus of FH will also cause the clinical presentation of aHUS by inhibiting FH self-recognition and binding, ablating the regulation of FH almost entirely.[187, 189] Curiously, some of the clinical manifestations of aHUS are similar to those of dengue e.g. vascular leakage and thrombocytopenia.

I-2.6.5 FH exploitation in infectious diseases

The AP is vital for eliciting an acute response against foreign cells and viruses by recognising non-self surfaces. Thus, avoiding, or negating C3b deposition on microbial surfaces improves a pathogen's ability to survive in the host. To their benefit, members of arthropod, fungi, bacteria, and families of virus have convergently evolved mechanisms of complement evasion by exploiting FH regulation of the AP.[190-192] For example, *Neisseria meningitidis* is a pathogenic bacterium that can cause meningitis and sepsis. *N. meningitidis* evades the AP by binding FH to its cell surface, through Neu5Ac and FH binding protein (FHbp) thus being recognised as 'self' by the AP **(Figure I-10)**.[193] The interaction between complement and *N. meningitidis* is described in greater detail in **Section VI.8**. Interestingly, meningococcal FHbp has been theorised to rapidly deplete circulating FH through sequestration, deregulating complement, and has been proposed as a mechanism of haemorrhagic rash in meningococcal sepsis.[193] This proposed mechanism of complement mediated vasculopathy is akin to the concept of DENV-mediated deregulation of complement AP described in detail in **Section 2.6.4**. Similar to *N. meningitidis*, C*andida albicans* is a yeast that also evades AP activation by recruiting circulating FH, preventing C3b deposition using surface attached *C. albicans* glycerol 3-phosphate dehydrogenase-2 (Gpd2) **(Figure I-10)**.[194] Mosquito gut ECs are also primed to bind FH by binding the

mid-gut endothelial glycocalyx, which protects from complement protein within blood meals. Without FH, the AP would promote lysis of the gut cells that whole blood comes into contact with **(Figure I-10)**. Human immunodeficiency virus (HIV) binds FH through the transmembrane protein GP41 **(Figure I-10)**. The potency of complement-mediated pathogen clearance is apparent in the example of HIV. Here, HIV binding of FH to GP41 reduces complement mediated virolysis in serum-incubated HIV by 80% but this effect is lost in the presence of GP41 monoclonal antibodies directed at the FH binding site.[191, 195] Examples of FH binding and mimicry for all kinds of parasites, converging on a similar theme of complement evasion emphasises the potency of FH control of the AP.[196]

I-2.7 The FH Family

A subset of FH protein isoforms mimic some SCR domains but not actions of full-length FH. It is thought that these FH related proteins (FHR) arose from gene duplications of FH less than 19 million years ago in primates.[185] The existence of these isoforms has emphasised the complexity involved in AP activity, where the regulatory molecule FH is itself thought to be regulated by FHR proteins.[197, 198]

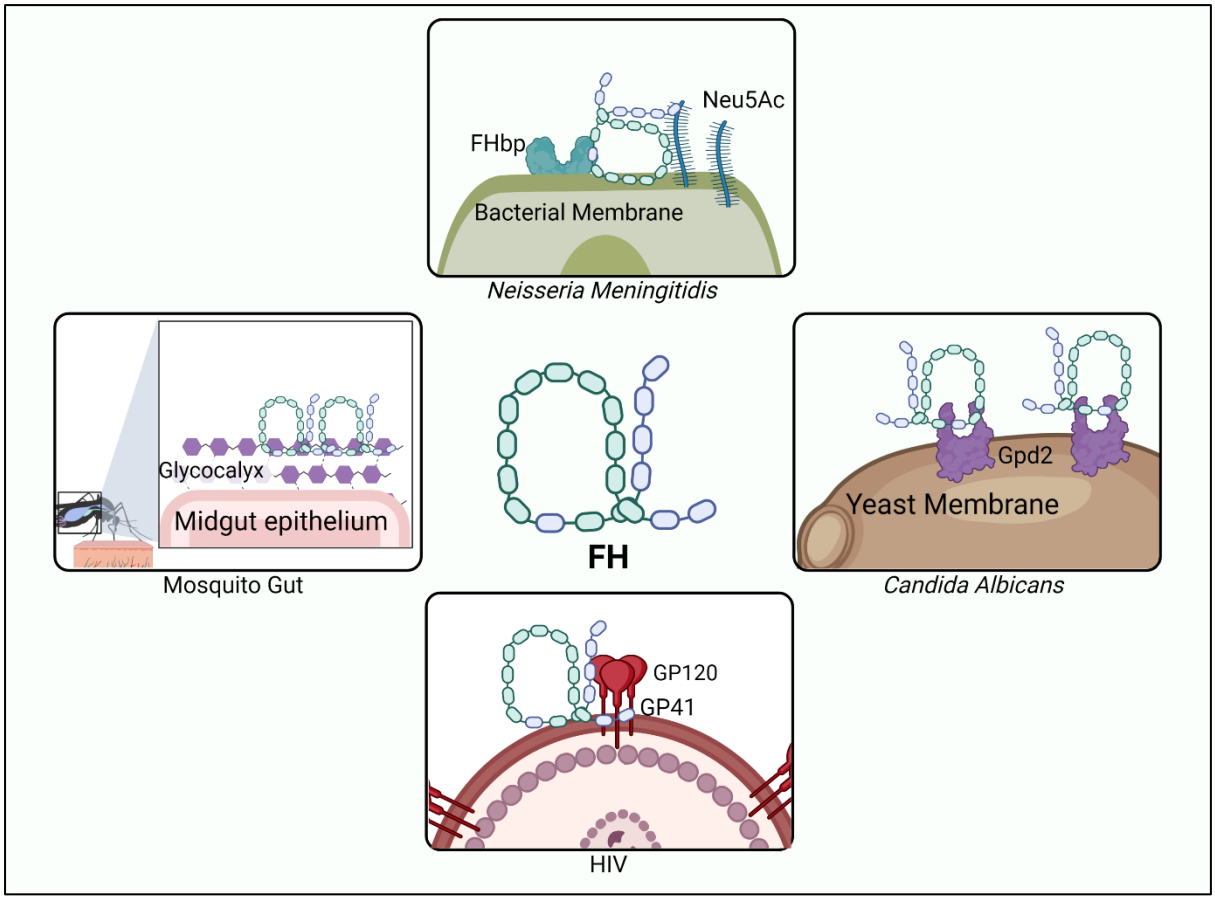

Figure I-10 Complement FH is acquired by all forms of parasites as a mechanisms of evading complement-mediated destruction.

Neisseria meningitidis expresses polyanionic Neu5Ac and the transmembrane protein FHbp to recruit FH to the bacterial surface. Mosquito midgut epithelium layers are highly glycosylated and recruit FH from ingested blood via GAGs and sialic acids to avoid complement mediated gut cell destruction during feeding. *Candida Albicans* acquires host FH by surface attached *C. albicans* Gpd2. HIV-1 uses the transmembrane protein GP41 to bind FH which protects from virolysis. FH = Factor H, Neu5Ac = N-Acetylneuraminic acid, FHbp = Factor H binding protein, Gpd2 = glycerol 3-phosphate dehydrogenase-2, GP = glycoprotein, HIV = human immunodeficiency virus.

I-2.7.1 The FH Related proteins

The FHR proteins are members of the RCA, and are composed entirely of complement control domains which resemble FH SCRs. The five canonical FHR proteins (FHR1-5), however, are transcribed from separate, individual genes *CFHR1, CFHR2 etc.* The FHR proteins were numbered in order of discovery and their assigned numbers do not align with their order along chromosome 1.[197] FHRs are less abundant than FH, where the most abundant FHR, FHR-1, is approximately 3-fold lower than FH in the blood accounting for weight (100µg:500µg FHR-1:FH).[197] Additionally, FHRs have been observed to both activate and regulate the AP**.** Therefore, FHRs seem to play distinct roles from FH, despite containing many similar motifs.[199] Excitingly, the FHR research field is rapidly expanding, where the roles FHR play in complement activation and infection have been demonstrated to fill niche roles that the full length FH molecule cannot.[197] In addition to new roles of the FHR proteins, discovery of native (homo and hetero) dimers and tetramers adds to the complexity of researching this protein family.[200-202] The interactions between the FHR and AP activity are summarised in **Figure I.11.**Similarities between FH and FHR1-5 complicate assay development when analysing either mRNA or protein, discussed further in **Chapter IV.**

I-2.7.2 FHR-1

FHR-1 refers to both the 37 and 43 kDa isoforms expressed from the *CFHR1* gene. FHR-1A and B are highly similar and contain SCRs that have retained moderate to high identity with the same domains of the parent FH protein. Notably, FHR-1$_{SCR3-5}$ has high identity to FH$_{SCR18-20}$ (95% 100% and 97%) **(Figure I.12)**.[197]

FHR-1 is clearly not essential for life, as millions of individuals possess deletions for one or both of *CFHR1*, which is accompanied by the concomitant deletion of *CFHR3* forming the genotype *CFHR3-1Δ*. This deletion curiously protects from AMD and is discussed in depth in **Chapter VI**.[203] FHR-1 deficient individuals have higher rates of aHUS due to autoantibodies that are elicited against the FH C-Terminus. It is theorised that this is due to similarities between FHR-1$_{SCR3-5}$ and FH$_{SCR19-20}$, which promotes greater tolerance for the common epitopes of the two proteins.[197, 204]

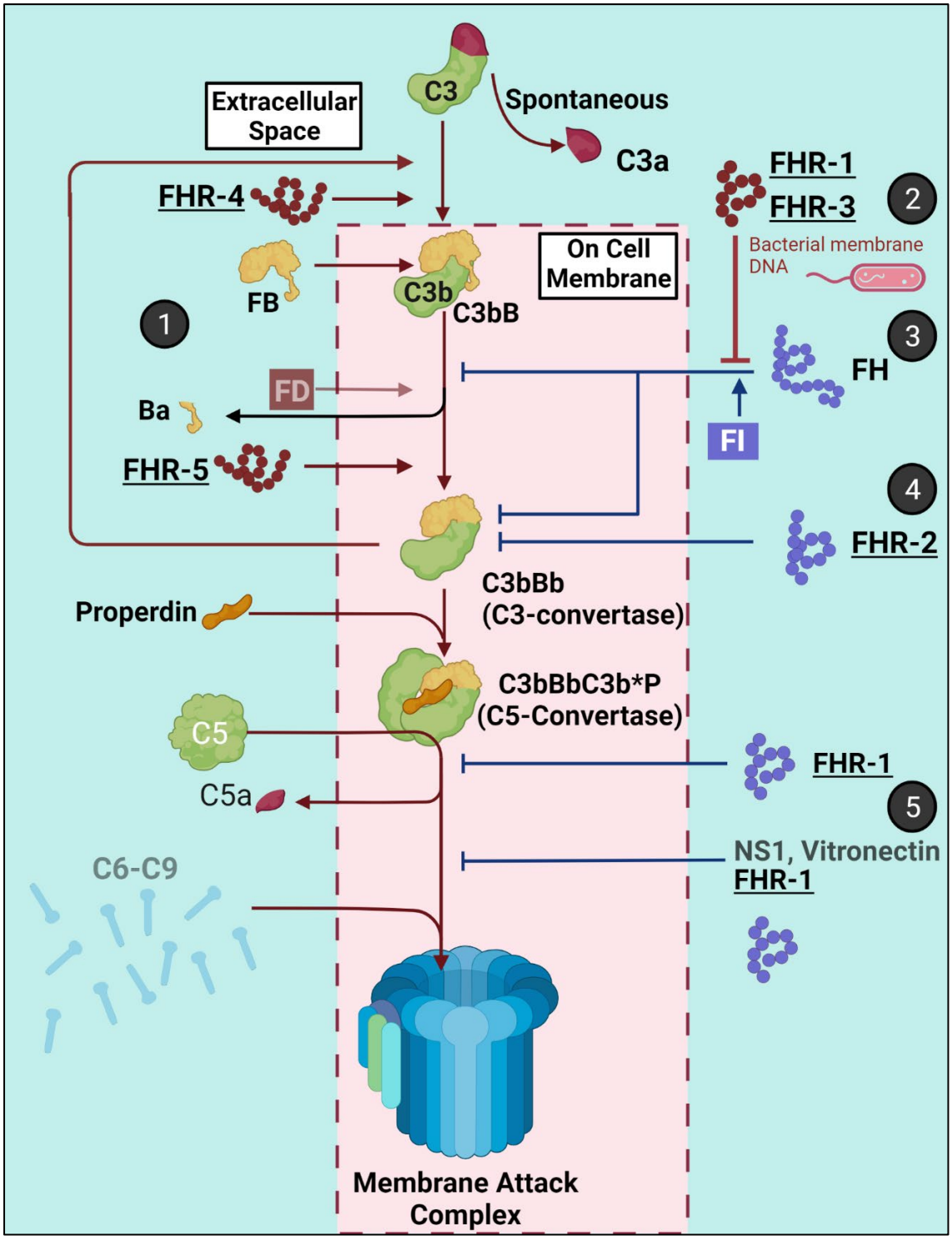

Figure I-11. The complement AP is both activated and regulated by FH and FHR-1-5.

(1) FHR-4 and FHR-5 promote AP activation by providing scaffolds for C3bBb convertase formation. **(2)** FHR-1 and FHR-3 compete with FH for C3b to prevent FH-mediated AP regulation on the surface of bacteria (FHR-1 and FHR-3) and extracellular DNA (FHR-1). **(3)** FH regulates early AP activation stages by binding C3b on the host cell membrane, preventing FB binding. FH also destabilises the C3bBb complex and causes irreversible dissociation. **(4)** FHR-2 does not compete for FH when binding the C3bBb complex and inhibits activity of the complex. **(5)** FHR-1 can inhibit C5-convertase activity and membrane attack complex formation. FHR molecules facilitating AP activity are depicted in red, FH and FHR molecules regulating the AP are depicted in blue. FH = Factor H, FHR = FH Related

FHR-1 has some complement regulatory ability, inhibiting C5-convertase activity and MAC formation but does not regulate earlier C3b generating processes like FH.[205] In fact, FHR-1-C3b binding will prevent FH-C3b binding the same molecule. FHR-1, will competitively bind many FH binding ligands on the surface of pathogens, which are attempting to subvert complement mediated destruction.[199] FHR-1 sequestration instead of FH is to the detriment of the pathogen.[199] FHR-1 and FHR-5 will also competitively bind components of the extracellular matrix of the host membrane and prevent FH-mediated regulation of complement on these surfaces, increasing inflammation and damage.[206] Thus, overall FHR-1 activity favours inflammation over complement regulation, despite the reverse being true for the parent FH molecule, likely protecting against infectious diseases at the expense of higher autoreactive complement.

I-2.7.3 FHR-2

FHR-2, exists in two forms which differ by glycosylation patterns.[207] The larger (29 kDa) and smaller (24 kDa) FHR-2 isoforms are referred to as FHR-2A, FHR-2B respectively.[207] Overall, the FHR-2 molecule contains only one region of high identity to FH, which is FHR-2_{SCR3-4} to $FH_{SCR19-20}$ (89%) **(Figure I.12)**.[208] FHR-2 will regulate the AP earlier than FHR-1, preventing C3 cleavage by the AP C3-convertase *in vitro* and has no recorded interaction or competition for FH or its ligands.[208]

I-2.7.4 FHR-3

FHR-3 is a FHR that does not regulate the complement AP. FHR-3 is 5 SCRs long with varying identity to the FH parent molecule. The identity of FHR-3 is high for the N-terminus FHR-3_{SCR1} to FH_{SCR5} (91%), FHR-3_{SCR2} to FH_{SCR6} (85%) but <65% identity is observed for FHR-3_{SCR3-5} **(Figure I.12)**.[209]

Although FHR-3 shares many ligands with FH, it will only compete with FH for C3b binding, without resultant inhibition of any AP stage. FHR-3, will also competitively bind FH-binding pathogens attempting to subvert complement mediated destruction, as in FHR-1, but the number of pathogens FHR-3 can bind is less than FHR-1.[199] As FHR-3 does not protect from AP activation, pathogens that acquire FHR-3 instead of FH are killed.[199, 210] As with FHR-1, FHR-3 is not essential for life, as homozygous deletion of *CFHR3* is present in millions of individuals.[203]

I-2.7.5 FHR-4

FHR-4 exists in two isoforms FHR-4A and FHR-4B (86 kDa and 42 kDa respectively) and no region of FHR-4 has identity to FH >70%. FHR-4 can initiate activation of the AP by providing a scaffold on cell membranes for C3b to bind. Resultant C3b binding can promote C3-convertase formation and so, implicates FHR-4 in complement activation.[211]

I-2.7.6 FHR-5

FHR-5 is 62 kDa, has the most SCRs of the five FHRs and the identity of FHR-5 SCRs to FH is low (50-75%).[212] **(Figure I.12)**. *In vitro,* CRP-coated to microtiter plates strongly bind FHR-5.[213] It was later confirmed that FHR-5 bound to necrotic HUVECs uses CRP binding to promote complement activation.[213, 214] Notably, a *CFHR5* mutation that causes FHR-5$_{SCR1-2}$ to be duplicated results in a dominant, toxic gain of function and is the cause of Cypriot *CFHR5* nephropathy.[215] Here, disease occurs due competitive binding of FHR-5 to nephritic C3b deposits, demonstrating the importance of access to C3b by FH.[215, 216] Thus, FHR-5 plays roles in activation of the AP which can also disrupt the ability of FH to regulate complement in genetic diseases.

As the field of FHRs expands, the role they play in homeostasis and infection seem to distinguish the roles of some FHRs from the roles of FH, despite initial assumptions suggesting they existed to compensate for the parent FH protein.

I-2.8 DENV and the complement system

DENV infection is impaired in the presence of CP and MBL proteins *in vivo* and correlates of protection from severe dengue have been found for the AP complotype of the individual.[182, 217-219] Importantly, if complement activation leads to exceedingly high anaphylatoxin concentrations, as can occur in response to infection, self-reactive damage can occur and worsen disease.[154, 220, 221] In immune cells, anaphylatoxin recognition will induce the proinflammatory cytokines correlated with worsened patient outcome (IL-1, IL-6, TNF-α, IL-8).[222-224] In dengue specifically, anaphylatoxin concentrations are inversely correlated to both platelet count and patient outcome. Thus, anaphylatoxins overall are thought to contribute to the haemorrhaging and hypovolemic shock caused by increased vascular permeability through promoting the release of inflammatory cytokines.[225] Complement activation in response to DENV infection is therefore important to investigate further due to its effects on vascular

permeability and excessive cytokine release that will result from poorly regulated complement activity.

I-2.8.1 DENV and the CP

CP proteins dramatically impair DENV's ability to infect cells. Although production of non-neutralising antibodies facilitates ADE for DENV, any form of bound antibody also promotes CP activation and opsonisation.[226] Thus, IgM and IgG that recognise and bind DENV induce CP-mediated clearance of the virus. Also, fewer antibodies bound to the virion are required to clear DENV in the presence of complement, demonstrating that the CP is able to enhance immunity against DENV.[227] In fact, complement provides protection from ADE to FC-γR-expressing cells by promoting complement-mediated phagocytosis.[228, 229] For example, Mehlop *et al*., (2007)[228] demonstrated in the presence of anti-DENV IgG, C1q reduced ADE by 43-fold in FCγRIIa-transfected K562 cells. In U937 cells, Yamanaka, Kosugi and Konishi (2008)[229] demonstrated a dose-dependent effect of C1q concentration that inhibited DENV-2 replicative ability in the presence of non-neutralising monoclonal mouse IgG raised against DENV-4.

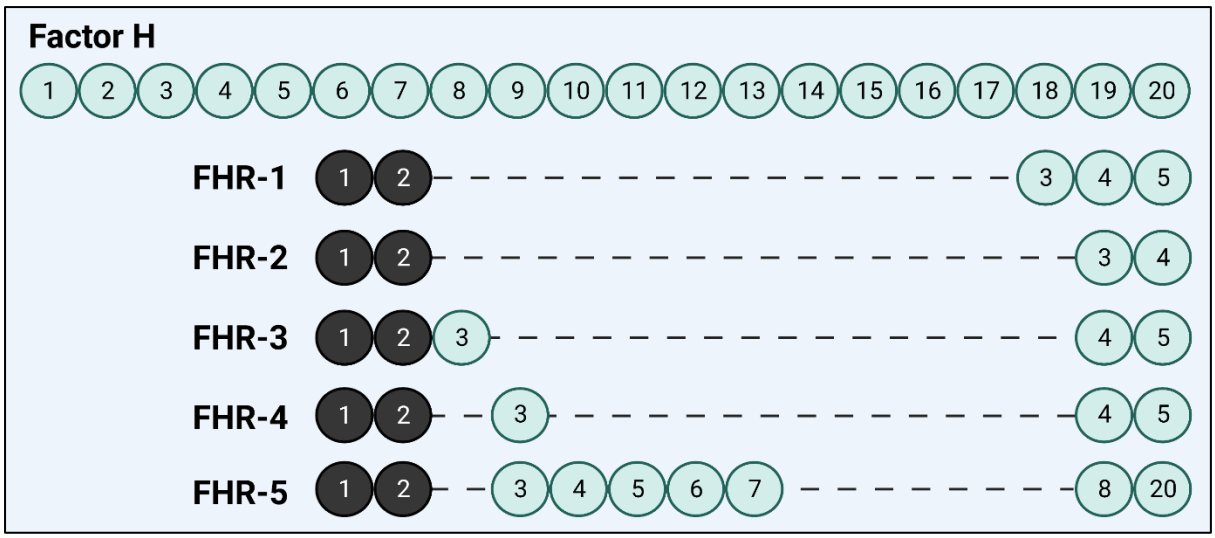

Figure I-12. The alignment of FH and FHR1-5 protein domains.

Factor H is a protein composed of 20 short consensus repeat domains which are also present in the FH gene duplication family, the FHRs. FHR-1$_{SCR3-5}$ is homologous to FH$_{SCR18-20}$. FHR-2$_{SCR3-4}$ is homologous to FH$_{SCR19-20.}$ FHR-3$_{SCR3}$ is homologous to FH$_{SCR8}$ and FHR-3$_{SCR4-5}$ is homologous to FH $_{SCR19-20}$. (FHR4B pictured) FHR-4$_{SCR1-3}$ is homologous to FH$_{SCR6, SCR8-9,}$ FHR-4$_{SCR4-5}$ is homologous to FH$_{SCR19-20}$. FHR-5$_{SCR3-7}$ is homologous to FH$_{SCR10-14}$ and FHR-5$_{SCR8-9}$ is homologous to FH$_{SCR19-20}$.

I-2.8.2 DENV and the MBL

MBL-binding initiates the lectin pathway cascade by recognising the mannose glycans present on the virion surface and can initiate destruction of DENV, independent of antibody binding.[219, 230] However, MBL-mediated viral clearance is more effective in mosquito derived virions than those produced by infected mammalian cells. Avirutnan *et. al.,* (2011)[219] demonstrated that the MBL pathway neutralises DENV, independent of membrane attack complex formation or antibodies. MBL-mediated C4 cleavage is primarily MASP-2 dependent.[231] Using serum from transgenic mice, Avirutnan *et. al.,* (2011)[219] also demonstrated that DENV-naïve wild-type mouse serum *ex vivo* neutralised three-fold more DENV than $MBL^{-/-}$ or $MASP\text{-}2^{-/-}$ mouse serum. Furthermore, in the same study, C6/36 mosquito cell-derived DENV fixed to microtiter plates was more efficiently bound by MBL than mammalian-derived DENV. This was attributed to differential mannose modification of the virion produced by mammalian cells.[219, 232] Therefore, the MBL is likely important for initiating complement activation against inoculating DENV and other arthropod-derived viruses, facilitating AP activation through C3b feedback mechanisms. However, the importance of the MBL decreases once replication in humans has begun, due to differences in host-dependent glycosylation patterns of nascent virus.

I-2.8.3 Evasion of the CP and MBL by DENV

DENV evades the complement system to infect the host through the use of the DENV non-structural protein NS1. NS1 synthesis in DENV infected cells results in secretion of the protein as a homohexamer, which can also bind back to the infected cell.[233] As a novel mechanism of immune evasion, DENV-NS1 can bind the complement control protein vitronectin. Vitronectin directly inhibits formation of the membrane attack complex, protecting DENV-infected cells from complement mediated destruction by the terminal pathway but not decreasing anaphylatoxin production.[234]

Additionally, mosquito-derived DENV-NS1 contains mannose glycans which bind MBL *in vitro*, although mannose decoration of human-derived DENV-NS1 is not as prominent. To investigate the effect of these glycans and their interaction with the MBL, Thiemmeca et *al.,* (2016)[235] demonstrated that MBL-NS1 binding was stronger in mosquito derived NS1 than NS1 produced by mammalian cells as determined by ELISA. The authors therefore concluded that mosquito-derived NS1 acts as a decoy, redirecting MBL activation towards NS1 and away from inoculating virions. Additionally, in this study, NS1 was demonstrated to bind the major regulator of the

CP and MBL pathways, C4bp, also determined by ELISA. In combination with C1s proenzyme, NS1-C4bp complexes degraded complement C4b on the surface of BHK21 cells, determined by decreased C4b and increased C4d (a C4b degradation product), and reduced complement activity, ultimately benefitting viral replication. It is clear that multiple complement evasion strategies of NS1 emphasise the threat that complement poses to DENV persistence in the blood.

I-2.8.4 DENV and the AP

Direct action of AP mediated DENV destruction has not been demonstrated. However, investigation by Avirutnan *et al. (*2006)[66] has shown that circulating NS1 can activate the AP independent of antibodies or the MBL. This implies NS1 can induce AP activation, potentially worsening complement-mediated disease resulting from DENV infection. FB expression is increased in response to inflammatory cytokines, increasing local AP activity.[236] Simultaneously, FH expression increases in response to inflammatory cytokines which limits off-target AP activation on host-cell surfaces. *In vivo* and *in vitro* analysis by our laboratory, of DENV infected MDM and EC have demonstrated that during DENV infection, only FB but not FH secretion increases, enhancing AP activity without AP regulation.[43, 217]

During the course of viral infection, infected cells develop altered cell surface profiles which can effect FH-cell binding.[237, 238] Importantly, FH regulation is mediated by binding of GAGs and sialic acids, as detailed in **Section I-2.6.1**, both of which are degraded on the endothelial glycocalyx by DENV-NS1. Puerta-Guardo et *al.,* (2016)[15] demonstrated increased activity of the protease cathepsin-L and heparanase and sialidase following NS1 treatment in the absence of DENV virions *in vitro*.[15] Here, proheparanase was converted to heparanase via cathepsin L (CTSL), and activation of sialidases NEU1-3 was observed for NS1-treated HUVECs within 4 hours. Activation of heparanase and sialidase acutely resulted in decreased HS and sialic acid observed by immunofluorescence (IF), and permeability measured by trans-endothelial electrical resistance.[15, 239] Thus, DENV-NS1 causes changes in the ligands for FH which may alter FH-membrane binding behaviours and thus, enzymes that interact with HS and sialic acid were investigated in **Chapter III**. Therefore, NS1 may indirectly increase AP activity on cell surfaces by reducing the number of HS and sialic acid sites for FH to bind. Accordingly, HS and sialic acid in DENV-infected cells were investigated in **Chapter IV.**

I-2.8.5 Increased FH abundance protects from severe dengue

Nascimento *et al.* (2009)[217] investigated AP protein concentrations from the sera of dengue and severe dengue patients. This study correlated decreased patient FH with decreased platelets and increased C3a and C5a, all correlating with worsened disease severity. Additionally, it was apparent that lower concentrations of factor D, which is a rate limiting factor of complement AP activation, was protective from severe dengue. *In vitro*, Dalrymple and Mackow (2012)[240] performed an array analysis of DENV-infected EC mRNA and observed 34-fold increased FB mRNA 48 hours post infection (hpi), showing DENV infection induces the proteins that activate the AP. This was later confirmed by our laboratory in MDM and HUVECs, demonstrating increased FB mRNA and secreted FB protein 48 hpi.[44] Conversely, in 1992, Shaio *et al.,*[241] demonstrated DENV-induced complement activation occurring by CP and MBL pathway activation, but concluded that the AP was not significantly more active during dengue. This is in direct contradiction of the above findings and observations by our laboratory. However, the method of assessing AP activity by Shaio *et al.,* was limited to defining the ability of blood from a limited number (n=9) of dengue patients to induce haemolysis in rabbit erythrocytes. Erythrocyte haemolysis assays are mainstay in assessing complement activity although, do not define the changes in abundance of mRNA or protein for any of the activators or regulators of the AP.

Through GWAS, a SNP in the *CFH* promoter region that increases circulating FH concentrations has been correlated with protection from severe dengue.[242] In a cohort of 121 Brazilian dengue patients (ages 0-76) rs375334 was detected, located in the NF-κB bindings site of the *CFH* promoter. The presence of rs375334 was corelated with a 20% increase in homeostatic circulating FH concentrations and a 2.5-fold decreased odds ratio for development of severe dengue for both primary and secondary infection. Another study investigating correlations of SNPs for *CFH*, *CFB* and *C3* and dengue severity did not observe this correlation or any other correlate of complotype with disease severity.[243] However, this study was conducted in a smaller cohort (n=61) of Thai children (2-15 years old), excluded primary infections, and therefore may have been affected by differences in ethnicity, cohort age or lower statistical power. Regardless, the complotype of individuals is not hinged on just one, but many, interacting proteins and may therefore compensate regulation of FH by other mechanisms.[244]

I-2.8.6 DENV infection disrupts FH secretion in vitro

Until recently, the induction of FH mRNA during DENV infection had not been defined. Using primary and immortalised cell lines, our laboratory has demonstrated that DENV infected MDM and HUVEC cells produced increased amounts of both FH and FB mRNA in response to DENV infection.[43]

In MDM, increased FH mRNA was observed after DENV and ZIKV infection **(Figure I-13A)**, but not increased FH protein in the supernatant **(Figure I-13B)**. FB mRNA was also increased in MDM by DENV and ZIKV infection **(Figure I-13C)** and FB protein was increased in the supernatant of DENV-infected cells but not ZIKV **(Figure I-13D)**. Therefore, increased FB mRNA and supernatant protein and FH mRNA not FH protein in the supernatant *in vitro* suggests DENV causes dysregulation of AP proteins in fluid phase distinct from ZIKV. Importantly, ZIKV is a flavivirus that does not induce vascular hyperpermeability. Furthermore, stimuli of host-antimicrobial pathways by the dsRNA mimetic, Poly I:C, and Gram-negative bacterial cell wall component, LPS, induced both FH mRNA and protein secretion. Therefore, DENV and ZIKV infection and not detection of their dsRNA genomes or activation of PRRs alone dysregulate the abundance of fluid phase FH.

Flow cytometry to quantify FH binding and DENV-infection in MDM and HUVECs demonstrated that DENV-infected MDM and HUVEC had higher percentages of FH positive cells than mock infected controls.[43] Additionally, MDM that were within the uninfected bystander cells in DENV culture had higher percentages of FH positive cells than mock infected MDM controls. FH binding to bystander HUVECs, however, was not different from mock infected HUVEC controls. These results demonstrated higher FH on the membrane of DENV-infected cells but not higher mean FH intensity. Therefore an increase in total FH protein was observed, possibly providing an explanation for unchanged FH in the supernatant for MDM, although not HUVEC, and FH-cell localisation still required further definition. These data also suggest that DENV infection causes some change on the cellular membrane that increases affinity of FH for DENV infected cells. Thus, gaps in the literature surrounding DENV-host-membrane augmentation and localisation of FH to the cell surface required investigation and were defined further in this thesis with a focus on FH cell surface ligands HS and sialic acid.

In consideration of existing patient data and *in vitro* evidence, it is apparent that investigations into the post-transcriptional regulation of FH during DENV infection is warranted. Intricate interactions of the complement system and importance of FH during dengue also supports the need to define changes in FHR1-5 abundance in response to DENV infection. To further define the relationship between FH and DENV, this thesis characterised induction of FH and FHR transcripts, augmentation of cell membrane carbohydrates, sialic acid and HS, and localisation of the FH protein during DENV infection.

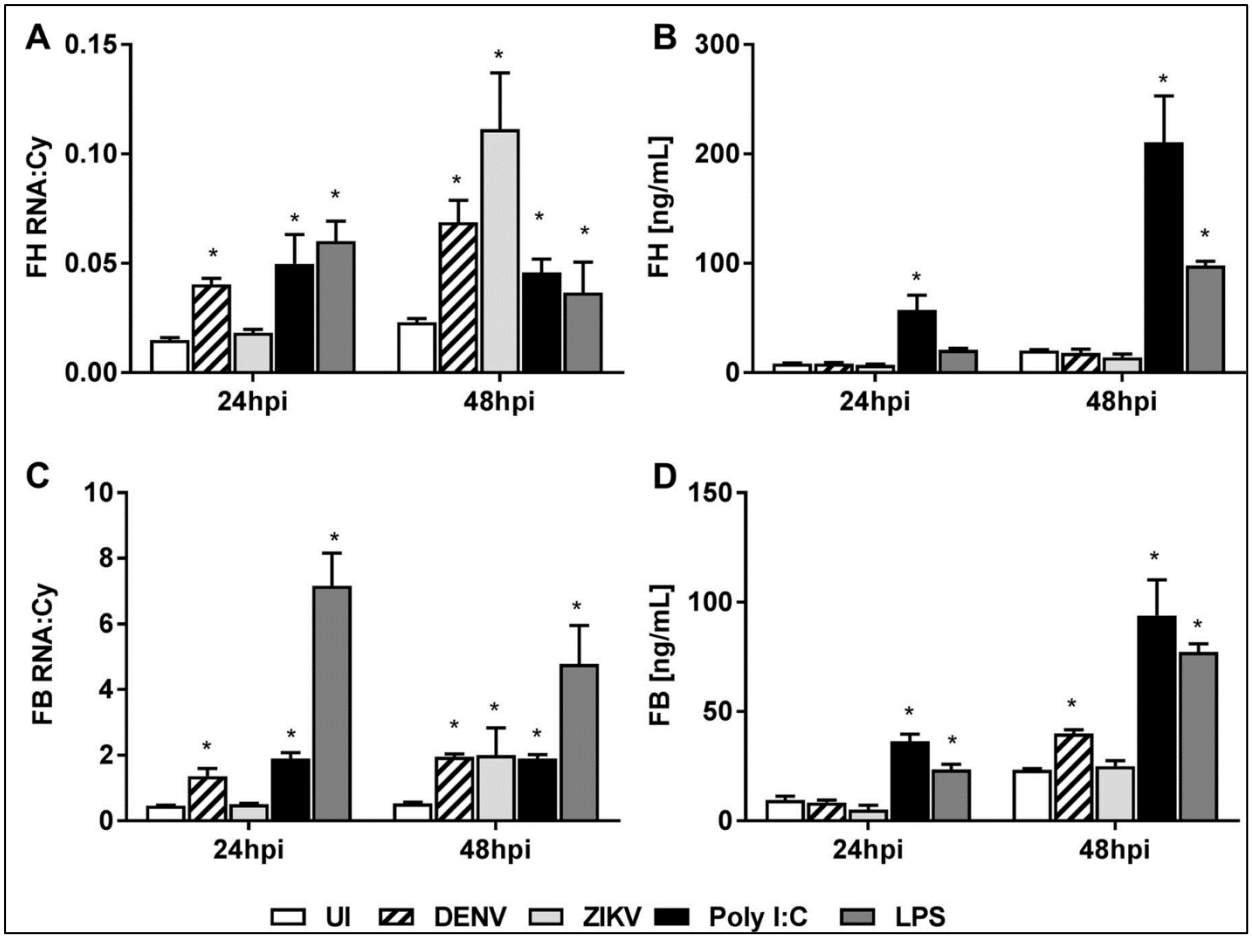

Figure I-13 TLR ligands of infection but not viruses produce both FH mRNA and secreted protein.

MDM were isolated and left uninfected or infected with DENV, ZIKV or stimulated with Poly I:C or LPS and **(A)** FH or **(C)** FB mRNA quantified by RT-PCR **(B+D)** and secreted protein by ELISA of the supernatant produced by the same cells. Results represent the mean and standard deviation for two samples and are representative of three biological replicates. *=p<0.05, one-way ANOVA and Tukey's t-test. MDM = monocyte derived macrophages, FH = Factor H, FB = Factor B, hpi = hours post infection, Cy=cyclophilin. Figure taken from Cabezas (2018)[43].

I-3 Hypotheses

Hypothesis and Aims 1

The literature surrounding correlates of dengue patient outcome and the complement AP clearly point to the contribution of dysregulated complement control in dengue disease severity. *In vitro,* DENV infection in primary MDM and HUVEC has revealed that infected cells have induced FH and FB mRNA transcription that is met with only elevated FB protein release from cells. This transcriptional discord caused an extracellular imbalance between the AP regulator (FH) and activator (FB), in the fluid phase as observed previously by our laboratory. Therefore **Hypothesis 1** proposes that:

> *Dengue virus infection induces full length factor H mRNA and protein. However, factor H is retained by the cell through cell membrane augmentation, rather than released into the extracellular space.*

Specifically, this study aimed to:

1. Determine if the pattern of FH mRNA induction in dengue and severe dengue patients can be observed in existing dengue patient datasets *in silico* **(Chapter III)**

2. Define changes in transcript abundance for HS and sialic acid-interacting enzymes in dengue and severe dengue patients using existing dengue patient datasets *in silico* **(Chapter III)**

3. Characterise the *CFH* and *CFHR* promoter elements *in silico* to predict which elements would be activated by DENV infection **(Chapter III)**

4. Characterise a cell line that will recapitulate the phenomena of induced FH mRNA but not secreted protein during DENV infection as seen in MDM and EC, as a model for studying this phenomenon with better reproducibility and efficiency **(Chapter IV)**

5. To define the products of transcription, translation, and cellular localisation of FH protein during DENV infection *in vitro* **(Chapter IV)**

6. To determine the molecules responsible for induction of FH mRNA and protein during DENV infection **(Chapter V)**

Hypothesis and Aims 2

The original focus of this thesis was to investigate the complement AP and DENV infection specifically. Through serendipitous events and with constraints on wet-lab research due to the COVID-19 pandemic, the development of a thesis chapter was prepared as a manuscript and is now under peer review. This chapter reviews the evidence link Indigenous health outcomes and the common gene deletion *CFHR3-1Δ*. This thesis chapter is distinct from the investigations involving DENV but investigates the roles of the family of FH proteins more broadly.

The subject of Indigenous genetic research requires understanding, collaboration, respect, and justification. Therefore, **Chapter VI** aimed to provide a foundation for investigating the Indigenous Australian complotype and its relation to autoimmune and infectious diseases. There is an unacceptable number of diseases that disparately impact the Indigenous Australian community relative to people with non-Indigenous ancestry. The prevalence of these diseases in the Indigenous communities is likely borne from the inequity of income, political oppression, and centuries of marginalisation. However, the rates of diseases recorded for Indigenous Australians mirrors other ethnicities. In these other populations, but not yet assessed in Indigenous Australians, is the common 84 kb deletion of the FHR genes *CFHR3* and *CFHR1* (*CFHR3-1Δ*). Following comparisons of the prevalence of FHR-3 and FHR-1 related diseases in other ethnicities and Indigenous Australian communities, it seems possible that *CFHR3-1Δ* is contributing to the disparity in prevalence and severity of diseases that occur in Indigenous Australians. Therefore, **Hypothesis 2** is that:

> *The patterns of infection and autoimmune disease in Indigenous Australians is indicative of the CFHR3-1Δ complotype observed in other ethnicities.*

This chapter aimed to:

(1) Review, collate and critique existing data for complement-associated autoimmune and infectious diseases in the Indigenous Australian population. Then:

a. Compare the data of disease prevalence in Indigenous Australian communities to (i) the non-Indigenous Australians from the same region (ii) and disease prevalence of other ethnic groups with *CFHR3-1Δ*
b. Evaluate the hypothesis that Indigenous Australian communities carry high frequencies of the *CFHR3-1Δ* haplotype.

CHAPTER II

MATERIALS AND METHODS

II.1 Materials

Table II- 1. Cell lines used in this project

Cell	Species	Organ/Tissue	Cell Type	Experiments	Media	Source
HeLa	Human	Cervical Cancer (Cervix)	Endothelial	DENV Infection	Adherent Cell Complete Media	ATCC® CCL-2™
Huh7.5	Human	Hepatoma (Liver)	Hepatocyte (IFN deficient)	DENV Infection	Adherent Cell Complete Media	Kindly provided by Professor Michael Beard
HEK293 c18	Human	EBNA1 expressing kidney cells	Epithelial/ Fibroblast like	DENV Infection	Adherent Cell Complete Media	Kindly provided from Professor Stuart Pitson
U937	Human	Epstein Barr Virus transformed Histiocytic lymphoma	Pro-Monocytic	DENV Infection	U937 Complete Media	ATCC® CRL-1593™
Vero	African Green Monkey	Kidney	Epithelial	Virus production	Adherent Cell Complete Media	ATCC® CCL-81™
C6/36	*Aedes Albopictus*	Larvae	Larval	Virus production	C6/36 Complete media	ATCC® CRL-1660™
BHK-21	*Mesocricetus auratus*	Kidney	Fibroblast	Virus production	BHK-21 Complete media	ATCC® CCL-10™

Table II- 2. Buffers and Reagents used in this project

Buffer/Reagent	Experiment Used	Contains	Manufacturer
Suspension culture media	Cell Maintenance	RPMI-1640	HyClone, Thermo Scientific (Supplied Sterile)
		100 U/mL Penicillin	
		0.1 mg/mL Streptomycin	
		1% (v/v) GlutaMAX Supplement	
Adherent cell media	All Cell Culture	DMEM	HyClone, Thermo Scientific (Supplied Sterile)
		100 U/mL Penicillin	
		0.1 mg/mL Streptomycin	
		1% (v/v) GlutaMAX Supplement	
		10% (v/v) Foetal Calf Serum (FCS)	
C6/36 Complete Media	Virus Amplification	Basal Eagle Medium	Gibco, Thermo Scientific (Supplied Sterile)
		1 x Non-essential amino acids	
		1mM Sodium pyruvate	
		10% (v/v) Foetal calf serum (FCS)	HyClone, Thermo Scientific (Supplied Sterile)
		10 mM HEPES	
BHK-21 Complete Media	Virus Amplification	EMEM	Gibco, Thermo Scientific (Supplied Sterile)
		10% (v/v) FCS	HyClone, Thermo Scientific (Supplied Sterile)

Buffer/Reagent	Experiment Used	Contains	Manufacturer
Coating Buffer (pH 9.6)	ELISA	50 mM Sodium carbonate	ChemSupply (CSA Scientific)
		50 mM Sodium bicarbonate	
		Made in reverse osmosis water	
Wash Buffer	ELISA	0.05% (v/v) Tween-20 in PBS	Sigma
DMMB Assay Reagent (pH 3.0)	Sulphated Glycosaminoglycan Quantitation	40 µM 1,9-Dimethyl-methylene blue	Sigma
		40 mM Glycine	
		0.01M Acetic Acid	
		25 mM NaCl	Univar (Ajax)
Loading Buffer (reducing) (pH 6.8)	SDS-PAGE	0.25 M Tris-HCl	Sigma
		8% (w/v) Sodium dodecyl sulphate (SDS)	
		0.05% (w/v) Bromophenol blue	
		0.05% (v/v) 2-Mercaptoethanol	
		20% (w/v) Glycerol	Univar (Ajax)
Running Buffer (pH 7.6)	SDS-PAGE	192 mM Glycine	Sigma
		0.1% (w/v) SDS	
		25 mM Tris-HCl	
1x BioRad Turbo Trans-blot	Western blot	5x Trans-Blot Turbo diluted in water and ethanol (See manufacturer's instructions: http://www.bio-rad.com)	BioRad

Buffer/Reagent	Experiment Used	Contains	Manufacturer
Tris Buffered Saline + Tween-20 (TBST) (pH 7.6)	Western blot	10 mM Tris	ChemSupply (CSA Scientific)
		50 mM NaCl	Univar (Ajax)
		0.1% (v/v) Tween-20	Sigma
Blocking Solution	Western blot	5% w/v skim milk powder in TBS-T	N/A
Blocking Solution	Immunofluorescence	1% (w/v) Analytical grade BSA	Sigma
		2% (v/v) Normal Donkey Serum	
		Made in PBS	
Luria-Bertani Broth (LB) pH (7.0)	Transforming DH5α and Maxiprep	5 g/L (w/v) Yeast extract	Gibco (Thermo Scientific)
		0.14 M Tryptone	
		0.34 M NaCl	Univar (Ajax)
LB Agar for plates (pH 7.0)	Transforming DH5α	5 g/L (w/v) Yeast extract	Gibco (Thermo Scientific)
		0.14 M Tryptone	
		15 g/L (w/v) Bacto Agar	
		0.34 M NaCl	Univar (Ajax)
Passive lysis buffer (PLB)	ELISA and Western Blot preparation	1x Passive lysis buffer	Promega
		1x Pierce protease inhibitor mini tablets, EDTA-free per 10 mL	Thermo Scientific
		Made in cell culture grade H_2O	Gibco, Thermo (Supplied Sterile)
0.5x TBE (pH 8.3)	Agarose Gel Electrophoresis	45 mM Tris	ChemSupply (CSA Scientific)
		45 mM Boric Acid	Merck
		1 mM EDTA	Univar (Ajax)

Table II- 3 PCR Primers used in this project. Aside from DENV, all primers were designed to bind human genes.

Primer Name	Accession Number(s)	cDNA Target	Sequences (Forward and Reverse)	Amplicon Size (bp)
SCR2	NM_000186.3	5' region of the FH transcript, **detects both FH and FHL-1**	F: AGGCCCTGTGGACATC R: AACTTCACATATAGGAATATC	182
Exon 9/10		Central region of FH Transcript, **detects only FH**	F: ACTCCCAGATGCATCCGTGT R: GGTTGAGCTGACCATCCATCTTT	173
SCR20		3' region of FH Transcript, **detects FH and FHR-1**	F: CATCCGTGTGTAATATCCCG R: TCTTTTTGCACAAGTTGGATAC	197
FHL-1	NM_001014975.2	Unique 3' UTR of FHL-1 Transcript, **detects only FHL-1**	F: CATCCGTGTCAGCTTTACCCT R: CATCCGTGTCAGCTTTACCCT	158
panFHR-1	NM_001379306.1	Both FHR-1A and FHR-1B isoforms are detected by these primers	F: GTACTGTGGGCGGATTCACA R: CTCTGTTGGGGGAGAAGAAAAATA	368
panFHR-3	NM_021023.6 NM_001166624.2	Both FHR-3A and FHR-3B isoforms are detected by these primers	F: ACCCCAGATGCGAATAAGGC R: TGGCTGTGTTCCCAATGCTC	630

Primer Name	Accession Number(s)	cDNA Target	Sequences (Forward and Reverse)	Amplicon Size (bp)
C3	NM_000064.4	C3 transcripts	F:GATGGTTACGGTCTGCTGGTGA R: GTGGAAATCCGAGCCGTTCTCT	132
FB	NM_001710.5	Factor B transcripts	F: ACTGAGCCAAGCAGACAAGC R: AGAAGCCAGAAGGACACACG	280
DENV	AF038403.1	DENV-2 capsid region of RNA (location 86-187)	F: GCAGATCTCTGATGAATAACCAAC R: TTGTCAGCTGTTGTACAGTCG	102
Cyclophilin	NM_021130.4	Transcripts for the housekeeping gene cyclophilin	F: GGCAAATGCTGGACCCAACACAAA R: CTAGGCATGGGAGGGAACAAGGAA	355

Table II- 4 Antibodies used for Immunofluorescence, ELISA, and Western Blot

	Antibody	Working concentration	Experiments	Supplier	Catalogue Number
Primary	Goat Polyclonal anti-human FH (43 µg/µL)	IF = 1:20 ELISA = 10 µg/mL Western blot = 1:2,000	Immunofluorescence, ELISA, Western blot	Calbiochem	341726
	Mouse Monoclonal anti-human FH	1:10,000	ELISA	Abcam	(OX23) or ab17928
	Mouse anti-dsRNA	1:200	Immunofluorescence	MozzyMabs	2G4
	Mouse anti-actin	1:2,500	Western blot	Millipore	MAB1501
Secondary	Rabbit anti-Goat horse radish peroxidase (HRP)	Western blot = 1:25,000	Western blot	Calbiochem	AP106P
	Rabbit anti-mouse HRP	Western Blot = 1:25,000 ELISA = 1:10,000	Western blot ELISA	Calbiochem	W4021
	Alexaflour 488 Donkey anti-mouse IgG H+L	1:200	Immunofluorescence	Thermo Scientific	A-21202
	Alexaflour 555 Donkey anti-goat IgG H+L	1:200	Immunofluorescence	Thermo Scientific	A-21432

Table II- 5 Genes and GenBank accession used for promoter analysis

Gene Name	GenBank Accession	Sequence location (NC_000001.11 Chromosome 1 ReferenceGRCh38.p14)
CFH	NG_007259.1	196,650,943-196,652,143
CFHR1	NG_013060.1	196,818,631-196,819,831
CFHR2	NG_042816.1	196,942,638-196,943,838
CFHR3	NG_015993.1	196,773,740-196,774,940
CFHR4	NG_028159.1	196,886,952-196,888,152
CFHR5	NG_016365.1	196,976,456-196,977,656

II. Methods

II.1 Cell maintenance

Cell culture was performed using aseptic technique in a class II biosafety cabinet. HEK293, HeLa and Huh7.5 **(Table II-1)** were passaged every three and four days, cultured in adherent cell culture media **(Table II-2)**. U937 cells **(Table II-1)** were passaged every three and four days, cultured in suspension culture media **(Table II-2)**. Cells were incubated in a humidified incubator at 37 °C and 5% CO_2. To passage adherent cells, cultures were treated with 2 mL 0.25% (v/v) trypsin (HyClone, Thermo Scientific) and incubated at 37 °C for 2-5 minutes (min) dependent on cell line. Trypsinisation was ceased by addition of 3 mL complete media **(Table II-2)** and cells harvested. For U937, cell media containing cells in suspension was harvested directly. An aliquot of harvested cells were mixed with 0.4% w/v trypan blue (BDH) and counted using a counting chamber (Hawksley). Following counting, $5x10^5$ cells were reseeded into a vented 75 cm^2 cell culture flask (Nunc, Thermo Scientific) in 17 mL of their respective media and incubated as described above. The calculation to determine cell density is as below:

$$Cells/mL = \frac{Dilution\ factor \times cells\ counted\ per\ square}{Chamber\ volume\ (mL)}$$

II.2 Viral infection

All infectious materials were handled in a class II biosafety cabinet in an OGTR-certified PC2 facility, and inactivated or transported in appropriate containment vessel before analysis. Use of DENV was approved for the laboratory by the IBC, in accordance with OGTR guidelines, prior to the commencement of this project (NLRD2011-10-7). DENV infections were performed with the laboratory DENV-2 clone, Mon601 derived from the New Guinea C strain.[245] Virus stocks were generated in BHK21 cells via transfection of *in vitro* transcribed RNA and then amplified in *Aedes albopictus* C6/36 cells **(Table II-1)**. Infected C6/36 supernatant was harvested, clarified, filtered, and then stored at -80 °C. Viral titres of infectious DENV were quantitated by plaque assay using Veros **(Table II-1)**. Virus stock generation and C6/36 supernatant preparation was performed by Associate Professor Jillian Carr.

Cells were seeded the day prior to infection with DENV at a multiplicity of infection (MOI) of 1 for 90 min at 37 °C. Following infection, inoculum was removed, cells washed and cultured in respective complete media **(Table II-2)** at 37 °C with 5% CO_2. Supernatants were harvested at 48 hpi and centrifuged at 8,000 x *g* for 1 min to clarify and then stored at -80°C prior to FH analysis by ELISA. Cells were either lysed in PLB **(Table II-2)** for ELISA and Western blot or in TRIsure (Bioline) for RNA extraction and RT-qPCR.

II.3 RNA extraction

TRIsure-preserved lysates produced in **Section II.2** were mixed with 200 µL of chloroform per 1 mL TRIsure and centrifuged at 4 °C for 15 min at 12,000 x *g* (Heraus Fresco 17 Microcentrifuge, Thermo Scientific). The aqueous phase was collected, and RNA precipitated in 1 mL 100% (v/v) isopropanol (Univar, Ajax) per mL of TRIsure for 10 min room temperature (RT). Samples were centrifuged again at 4 °C for 15 min at 12,000 x *g.* Isopropanol was discarded and the RNA pellet was washed with 1 mL 70% (v/v) ethanol (Chemsupply, CSA Scientific) and centrifuged for 5 min at 7,500 x *g.* The ethanol was discarded and the tube containing the RNA was air-dried for 10 min. The RNA pellet was then dissolved in 20 µL of nuclease free water.

II.4 DNA removal by DNAse I

To remove contaminating residual genomic DNA from the RNA extracted in Section **II.3**, 10 IU of DNase I (NEB) and 1X DNase I Reaction buffer (NEB) were added to the RNA and incubated at 37 °C for 15 min. The DNase reaction was stopped by addition of 5 mM EDTA (Sigma Aldrich) and heating samples to 75 °C for 10 min. Total RNA was quantitated using a NanoDrop Lite (Thermofisher) as per manufacturer's instructions. RNA integrity was assessed using the absorbance ratio at 260 nm and 280 nm, and RNA immediately stored at -80 °C.

II.5 Reverse transcription PCR

RNA from **Section II.4** was reverse transcribed into cDNA for use in qPCR. In brief, 500 ng of RNA was incubated with 30 µM random hexamers (NEB), diluted in RNase free-water (NEB) at 65 °C for 5 min and then rapidly cooled to 4 °C using a GeneAmp PCR System 9700 (PE Applied Biosystems) thermocycler. Next, 10 U of M-MuLV reverse transcriptase (NEB), 500 µM dNTPs (NEB), 10 U human placental RNase inhibitor (NEB) and 1X M-MuLV reaction buffer (NEB) were added to the primed RNA

and incubated at 37 °C for 90 min then 95 °C for 5 min and snap cooled to 4 °C by thermocycler, the cDNA was then diluted 1:2 with nuclease free H_2O and stored at -20 °C.

II.6 Real time quantitative PCR

Real time quantitative PCR was performed on cDNA produced from **Section II.5** using primers corresponding to the genes of interest found in **Table II-3**. All qPCRs were performed using the following ratios of reagents and cycled on a Rotor-gene 6000 real-time PCR cycler (Qiagen). First, 2 µL of samples were mixed with 5 µL iTaq SYBR green (BioRad), 0.5 µL each of 20 µM forward and reverse primer, 2 µL of nuclease free water. PCR reagent contamination was assessed in every run by inclusion of a no template control. Samples were heated to 95 °C for 5 min and then cycled 40 times at: 95 °C for 15 seconds, 59 °C for 30 seconds, 72 °C for 30 seconds. Samples were then heated once at 72 °C for 5 min. The FH plasmid cloned by Blackmore *et al.,* (1996)[247] (500 fg-500 pg serially diluted 10-fold) generated in **Section II.20** was used as a standard curve. qPCR values were expressed as the Ct values, normalised to the housekeeping gene cyclophilin and untreated/uninfected controls according to the double delta Ct method.[248]

II.7 Protein quantitation assay

Lysates prepared for protein quantitation and ELISA contained virus lower than the detectable limit of plaque assay. Therefore, PLB-treated samples were handled outside of the class II biosafety cabinet.

Lysates were diluted twenty-fold using PBS. 5µL of the diluted samples was added to a 96-well plate alongside bovine serum albumin (BSA) protein standards (0.05-1 mg/mL) and all samples received 200 µL of 1x Bio-Rad Assay Protein Assay Dye Reagent (stock diluted 1:4 in reverse osmosis water). Samples were left to incubate at RT for 15 min and then absorbance of samples was measured using a Spectramax® ID 5 (Molecular Devices) at 595 nm as per manufacturer's instructions. BSA standards were used to draw a standard curve (R^2>0.990) and sample concentrations determined using the equation of the line.

II.8 FH ELISA

The following protocol for the FH ELISA was devised and validated by Dr Sheila Cabezas, (2018).[43] Goat anti-human FH **(Table II-4)** was coated onto medium-binding 96-well plates (Greiner) at 10 µg/mL diluted in ELISA coating buffer **(Table II-2)** and incubated overnight at 4 °C in a humidified container. The antibody solution was removed and then wells blocked with 200 µL 2% (w/v) BSA (Sigma Aldrich) in PBS. The plate was then incubated for 1 hr at 37 °C. Blocking buffer was removed and 100 µL of samples were added at previously determined dilutions (1 in 4 for supernatant, 1 in 2 for lysates, in 1% BSA (w/v)). Samples were then incubated in the plate for 1 hr at 37 °C. Unbound proteins were removed from the plate and rinsed three times with ELISA wash buffer. Mouse anti-human FH (100 ng/mL) **(Table II-4)** was then added to each well and incubated for 1 hr at 37 °C. The unbound mouse anti-human FH was then removed by washing as above. Goat anti-mouse IgG (1:10,000) conjugated to horse radish peroxidase (HRP) **(Table II-4)** was then added to each well and incubated for 1 hr at 37 °C. Goat anti-mouse was washed as described above. The colorimetric reaction was developed by adding 200 µL tetramethylbenzidine peroxidase substrate (KPL) and incubating at RT for 5 min. The reaction was stopped using 100 µL 1M H_2SO_4 (Chemsupply, CSA Scientific). Absorbance at 450 nm was determined using a Spectramax® ID5 and FH concentration determined using known values (80 ng/mL to 2 µg/mL) of a standard curve from purified human FH. FH was purified from human sera by Dr Sheila Cabezas by column-based purification as described in Cabezas (2018).[43]

II.9 SDS-PAGE and Western blot

Lysates harvested from uninfected and DENV-infected cells were boiled with SDS loading buffer **(Table II-2)** at 90 °C for 10 min. Samples were loaded onto a 10% precast midiprotein gel (Criterion) alongside the pre-stained Kaleidoscope molecular weight ladder (Bio-Rad). The gel was electrophoresed for approximately 20 min at 300 V then transferred to a PVDF membrane (Bio-Rad) using the BioRad Trans-Blot Turbo system as per manufacturer's instructions. The membrane was blocked with 5% (w/v) skim milk powder in TBST **(Table II-2)** for 1 hr and then incubated overnight with either goat-anti-human FH (1:2,000) or mouse-anti human actin (1:2,500) **(Table II-4).** The membrane was then incubated with either rabbit anti-goat IgG HRP (1:25,000) or rabbit anti-mouse IgG HRP (1:25,000) **(Table II-4)**. Bound complexes were detected

by addition of ClarityTM substrate (Western ECL) and chemiluminescence captured using ChemiDoc™ MP Gel Imaging System (BioRad). Western blots were performed in the Flinders Proteomics Facility with assistance from Ms Nusha Chegeni and Ms Kamelya Aliakbari.

II.10 Proteasome inhibitor treatment

Cells infected as in **Section II.2** had media removed at 24 hpi and replaced with complete media containing either 5 nM Bortezomib, 5 μM MG132 (each kindly supplied by Professor Stuart Pitson, Centre for Cancer Biology, University of South Australia) or vehicle control (0.1% (v/v) DMSO in complete media). Cells were incubated for a further 24 hr at 37 °C, 5% CO_2 before harvesting supernatant for ELISA or cells lysed in TRIsure for RNA extractions in **Section II.3**.

II.11 Immunostaining for IF microscopy

$2.5x10^5$ HeLa were seeded onto coverslips in a 6-well plate and uninfected or DENV infected for 48 hr as in **Section II.2.** Cells were then washed with PBS three times and fixed with chilled 4% (w/v) paraformaldehyde (PFA) for 30 min at 4 °C. PFA was washed from cells with chilled 70% ethanol (v/v) and then with 1x PBS. Cells were then permeabilised by incubation for 15 min at RT in a 0.5% (v/v) IGEPAL® CA-630 (Sigma) solution. Coverslips were treated with IF blocking solution **(Table II-2)** and incubated at RT for 30 min. Permeabilised cells were probed with primary antibodies by incubating at 1:50 and 1:200 for goat anti-FH and mouse anti dsRNA respectively **(Table II-4)** in a humidified chamber at 4 °C overnight. Cells were washed three times with PBS and incubated with Alexaflour 488 donkey anti-mouse IgG and 555 donkey anti-goat IgG **(Table II-4)** and concomitantly stained with Hoechst-33342 (1mg/mL) (Thermo Scientific) for 1 hr at RT in a light proof chamber. The cells were then washed in PBS as above and briefly dried, before being mounted onto on glass slides with Prolong Gold Antifade Mountant (Invitrogen) and left to cure in the dark, overnight at 4 °C. Coverslips were sealed on slides with nail polish and viewed using either an AX70 fluorescence microscope (Olympus) or LSM880 Confocal Microscope (Zeiss). Images were captured using Zen Blue 3.0 software (Zeiss) and MP4s generated using Imaris (Oxford Instruments). Imaging and processing was performed in the Flinders Microscopy Facility.

II.12 Cleavage of cell surface HS and sialic acid

Cells were cultured and infected with DENV or left uninfected as described in **Section II.2**. At 48 hpi supernatants were removed and replaced with DPBS containing 5 mM $CaCl_2$ with or without 400 μ international units (μIU)/mL Heparinase I (Sigma Cat# H2519) and 415 μIU/mL Heparinase III (Sigma Cat# H8891). Cells were then incubated for 3 hr at 37 °C in 5% CO_2. Following incubation, cells were treated with DPBS containing 5 mM $CaCl_2$ or with DPBS containing 5 mM $CaCl_2$ and 90 mIU/mL neuraminidase (*Clostridium perfingens*; Sigma Cat# N2876). Cells were incubated for a further 1 hr. Supernatants were harvested for analysis of FH by ELISA as described in **Section II.8**. The cleavage of GAGs was independently confirmed through UV spectrophotometric analysis of cell supernatant by measuring absorbance at 280nm by Spectramax ID5, on a UV-transparent microplate (Nunc, Themo Scientific) and dimethyl methylene blue (DMMB) assay described in **Section II.13**. Sialic acid cleavage in HeLa treated with 90 mIU/mL was separately validated by the sialic acid fluorescence assay described in **Section II.14**.

II.13 Dimethyl methylene blue assay for quantitating sulphated GAGs

Dimethyl methylene blue (DMMB) reagent was prepared as described by Coulson-Thomas and Gesteira but with the amendment of adding 2M Tris (20% v/v final volume) prior to the reaction.[249] Immediately prior to the reaction, one-part 2 M Tris (pH 7.2) was added to four parts DMMB reagent **(Table II-2)**, then 200μL of activated DMMB reagent was reacted with 20 μL of chondroitin sulphate (Sigma, Cat# C9819) standard, undiluted supernatant, or cell lysate in a clear 96-well plate. Chondroitin sulphate was used to validate the assay amendments and then later as a standard from 30 μg/mL to 500 μg/mL. Passive lysis buffer, DMEM containing 10% FCS were used as negative controls. Absorbance at 525 nm was measured immediately by spectrophotometry (Spectramax ID5, Molecular Devices) and values determined by comparison to the standard curve.

II.14 Sialic acid fluorescence assay

Cells were seeded into a 96-well opaque-walled plate (Perkin Elmer cat# 6055300) at a density of $3x10^4$ cells/well in complete media and incubated overnight at 37 °C, 5% CO_2. Following incubation, cells were either uninfected or DENV infected (MOI=1) as in **Section II.2** and incubated as above. Validation that fluorescence was sialic acid dependent was performed below with the addition of serially diluted neuraminidase

(*C. perfingens)* (2-fold dilutions 90 mIU/mL to 3 µIU/mL) in DPBS containing 5 mM $CaCl_2$ one hr prior to fixing.

Cells were fixed 48 hpi by treatment with 4% (w/v) PFA for 30 min at 4 °C. Cells were stained with FITC-conjugated *Sambucus nigra* sialic acid binding lectin (25µg/mL) (Thermo Scientific) for 20 min in a light proof chamber. Cells were then washed 3 times with PBS and permeabilised for 20 min with 0.05% IGEPAL. Cells were then stained with Hoechst-33342 (1mg/mL) for 20 minutes in a light proof chamber. Cells were then washed 5 times with PBS, as above. Fluorescence at $495_{ex}/519_{em}$ was measured and normalised to $361_{ex}/497_{em}$.

II.15 Supernatant harvest for HeLa stimulation experiments

HeLa were seeded at a density of $1x10^6$ cells in a 75 cm^3 vented flask in complete media and incubated for 24 hr at 37 °C, 5% CO_2. Following incubation, cells had media removed and were either inoculated with DENV prepared as in **Section II.2** (MOI=1) in 5 mL DMEM or received 5 mL DMEM only, rocking every 15 min for 90 min. Inoculum was then removed and cells received 20 mL of complete media. Cells were left to incubate for 48 hr at 37 °C, 5% CO_2. Following incubation, cell culture media was harvested, and cells lysed in TRIzol for RT-qPCR to confirm DENV infection. Harvested supernatant was clarified by centrifugation at 2,600 x *g* for 5 minutes before size based fractionation in **Section II.16**. Conditioned media is the term given to the supernatant of cells generated in the absence of DENV-infection.

II.16 Sized based fractionation of DENV-infected HeLa supernatant

Supernatants of DENV-infected cells were either unfiltered or fractionated into large (>50 kDa) and small (<50 kDa) proteins by Ultra-15, MWCO 50 kDa centrifugal filters (Amicon) for 15 min at RT, at 2,600 x *g*. Supernatant fractions were then reconstituted to the starting volume (20 mL) in complete media. Supernatants and fractionated supernatants were stored at -80 °C until required for the stimulation of HeLa in **Section II.17**.

II.17 Using DENV-infected HeLa media to stimulate cells

HeLa were seeded the day prior to stimulation at a density of $1x10^6$ cells per well in a 6-well plate (Nunc) for RT-qPCR or onto coverslips for IF microscopy. The following day, cells had media replaced with conditioned media, unfiltered supernatant of DENV

infected HeLa, the large (greater than 50 kDa) or small (less than 50 kDa) fraction of infected cell supernatant generated in **Section II.16** or were infected with DENV (MOI=1) as in **Section II.2**. Cells were incubated at 37°C in 5% CO_2 and harvested at 8 hpi in TRIzol and stored at -80 °C for RT-qPCR analysis of DENV, FB, C3 and FH mRNA or fixed 24 hpi in 4% (w/v) PFA and prepared for IF microscopy as in **Section II.11**.

II.18 Serum free fractionated supernatant generation and mass spectrometry analysis

For mass spectrometry analysis, HeLa were infected in a 75 cm^3 vented flask as described in **Section II.15**, in the absence of FCS and DENV-infected supernatant was fractionated as in **Section II.16**. All samples were treated with Triton X-100 (0.05% v/v) (Sigma) to inactivate infectious virus, as previously validated by Associate Professor Jillian Carr by plaque assay. Supernatant protein was quantitated by BioRad protein assay described in **Section II.7**. 10 µg of protein from supernatants was prepared by Sera-Mag Carboxylate SpeedBeads (Cytiva) clean up as per manufacturer's instructions to remove contaminating salts and detergents. Protein was then digested using 0.5 µg trypsin gold (Promega Corporation, Alexandria, NSW, Australia) overnight at 37 °C. Peptide clean-up was performed for further salt removal using Sera-Mag Carboxylate SpeedBeads which were then dissociated from peptides as per manufacturer's instructions in preparation for analysis.

Following sample preparation above, samples were provided to the Flinders Proteomics Facility for analysis. Mass spectrometry (TSQ Altis™ Triple Quadrupole Mass Spectrometer, Thermo Scientific) was performed by Dr Alex Colella as previously described by Sharma *et al.,* (2009).[250] Protein species were identified using Thermo Proteome Discoverer software. Spurious protein values (peptide summary score <5) were removed from consideration of the results due to the high probability of error in identification of a protein. Abundance and summary peptide scores were then recorded for extracellular and secreted proteins. To determine relative protein abundance between datasets, raw abundance was first normalised to total protein detected. Raw data were made publicly available by submission to:

https://zenodo.org/record/6911339#.YuDXjHZByUl

II.19 Transforming and culturing bacteria for DNA amplification

Competent *Escherichia coli* strain DH5α (NEB) were thawed from -80 °C on ice for 30 min. 50 ng of the FH coding sequence plasmid "H20BS" cloned by Blackmore *et al.,* (1996)[247] was mixed with 50 µL of competent DH5α and incubated for a further 30 min on ice. Cells were then heated at 42 °C for precisely 1 min and snap-cooled on ice for 2 min. Cells were then cultured in 2 mL super optimal broth with catabolite repression media (Thermo Scientific) with ampicillin (50 µg/mL) in a 10 mL conical base tube for 1 hr, in an orbital incubator (Gallenkamp) at 37 °C. Transformed bacteria were streaked onto a LB agar plate with ampicillin (50 µg/mL) **(Table II-2)** and incubated overnight at 37 °C. Individual colonies were then cultured in 2 mL LB with ampicillin (50 µg/mL) in 10 mL tubes in an orbital incubator overnight at 37 °C rotating at 110 RPM. A 500 µL aliquot of the DH5α culture was inoculated into 250 mL of LB containing ampicillin (50 µg/mL) in a 2 L Erlenmeyer flask and then incubated again in an orbital incubator as above. Amplified plasmids were harvested by maxiprep.

II.20 Maxiprep

The bacterial culture from **Section II.19** was centrifuged at 3,400 x *g* for 10 min using a Sorvall RC 6 Plus centrifuge (Thermo Scientific). Plasmid DNA was extracted using an endotoxin free Maxiprep kit (Sigma Aldrich), as per manufacturer's instructions. In brief, pelleted bacteria were lysed and genomic DNA and proteins precipitated. Plasmid DNA was then column purified and eluted in 800 µl of nuclease free water. DNA purity and concentration were determined using Nanodrop™ Lite Spectrophotometer (Thermo Scientific) with an A260/280 between 1.7 and 1.8 and yields in the order of 1.6-2.3 µg/µl. The plasmid was confirmed to be the correct size by restriction enzyme digest and agarose gel electrophoresis described in **Section II.21-22**.

II.21 Restriction digest

Following maxiprep, 5 µg of FH20BS plasmid DNA from **Section II.20** was mixed with 40 units of SacI and KpnI (NEB), 2 µL of 10x NEBuffer 1.1 (NEB) and DNase free H_2O, added to a total volume of 20 µL. Samples were then incubated at 37 °C overnight and immediately used for agarose gel electrophoresis described in in **Section II.22**.

II.22 Agarose gel electrophoresis

An agarose gel (agarose (Progen) (2% w/v) in 0.5x TBE) was cast and submerged in 0.5x TBE **(Table II-2)** in an electrophoresis tank (Mini-Sub Cell GT Cell). Plasmid DNA

diluted to 3 ng/µL in 10 µL of deionised water or undiluted PCR samples were mixed 5:1 DNA to EZ vision III (AMRESCO Inc.), respectively and loaded alongside 100 bp DNA Ladder (NEB) for qPCR products and a 1 kb Plus DNA ladder (NEB) for plasmids. DNA was electrophoresed for 45 min at 90 V and 120 mA and viewed using a ChemiDoc imaging system (Bio-Rad).

II.23 *In silico* analysis of DENV patient microarray data using GEO2R

Gene expression array data was analysed using the NCBI array-analysis tool GEO2R and microarray data from whole blood of febrile and DENV-infected patients at different stages of infection and differing disease severity using the NCBI GEO database available at https://www.ncbi.nlm.nih.gov/gds. Statistical significance was determined using Benjamini and Hochberg adjusted p-values. Samples with adjusted p-values <0.05 were represented graphically as the fold-change from control data. Studies were included on the basis they contained patient microarray data and used probes to detect mRNA from *RSAD2* and *CFH*. Studies and data subsets were excluded from analysis if patients had received therapeutic intervention other than the standard of care.

II.24 PROMO *in silico* promoter analysis of the *CFH* gene family

The PROMO (Alggen) promoter analysis tool was used to predict the transcription factor binding sites in the regions upstream of *CFH* and *CFHR1-5* -1100 to 100 bp from the transcription start site. The 1.2 kb sequences were manually selected by locating *CFH* and *CFHR1-5* using NCBI GenBank **(Table II-5)** and analysed by PROMO available at http://alggen.lsi.upc.es/, with a maximum tolerance of dissimilarity threshold of 5%.[251] Outputs were tabulated and raw data files available at https://zenodo.org/record/6911659#.YuDZDHZByUk. The number of sites for a transcription factor binding element occurring by chance was determined as:

$$\text{Predicted number of sites by Chance} = \frac{1}{4^{(site\ length)}} \times 1200$$

$$\text{For sites with more than one possible nucleotide/site a fractional value is assigned}$$

$$e.g.\ \text{TT[G/A]TTTTG would have: (site length)} = 7.5$$

II.25 Statistics

Statistical analyses were performed using Prism 9 (GraphPad). One-Way ANOVA followed by Welch's *t*-test was used to determine significant differences between

samples in the proteasome inhibitor and GAG lyase experiments. Student's *t*-test were performed for the remaining data. Double delta transformation of RT-qPCR results, as described in Livak and Schmittgen (2001)[252], was used to determine changes in mRNA abundance. All graphs are representative of average values ± SEM and significance determined for $p<0.05$.

CHAPTER III

MINING PUBLICLY AVAILABLE MICROARRAY DATA FOR TRANSCRIPTIONAL CHANGES IN GENES THAT AFFECT FH REGULATION

III.1 Introduction

The complement AP causes devastating autoreactive damage if not properly regulated. In dengue patients, increased markers of AP activation in circulation are associated with life-threatening severe dengue.[217] Previously, a discord between FH and FB expression in response to DENV infection was observed for MDM *in vitro*, where DENV induced mRNA for FH and FB.[44] This opposes observations in dengue patients, where FH abundance is not different and even lowered in dengue and severe dengue respectively.[217] RNA was not isolated from these patients, so it is unclear if FH mRNA was induced in circulating cells, as it is in primary macrophages *in vitro*.[44] Therefore, It was hypothesised that FH mRNA would be induced in circulating cells from dengue patients, as observed previously by our laboratory *in vitro* and DENV-infected AG129 mice.

Further, FH is the parent molecule of a family of FH related (FHR) proteins, with 9 FH isoforms transcribed from the 6 FH-associated genes (FH, FHL-1, FHR-1A, FHR-1B, FHR-2, FHR-3, FHR-4A, FHR-4B, FHR-5) **(Figure I.11)**.[253] FHRs have not yet been investigated in the context of DENV infection, either in patients or in cells infected *in vitro*. FHR-1-5 each play a role in the complement AP, but some roles can oppose the FH parent molecule, which is the most important regulator of the complement AP.[198] For example, FH and FHR-2 similarly regulate AP at early phases of activation, however, FHR-4 and FHR-5 can promote AP activity.[254, 255] FHRs are also involved in immune surveillance and clearing cell debris, with their physiological importance becoming increasingly appreciated as our understanding of their roles in disease and immunity rapidly expands.[204, 238, 256-258] Due to their influence on the AP, FHRs may play a role in AP activity and regulation during dengue.

In addition to FHR proteins, other non-complement components can affect regulation by FH and thus, AP activity. Molecules of interest include proteins that anchor, cleave and degrade the primary ligands for FH on host membranes i.e. HS and sialic acid, such as proheparanase (HPSE), heparan-alpha-glucosaminide N-acetyltransferase (HGSNAT), heparan sulphate proteoglycan 2 (HSPG2), neuraminidases (NEU1-4) and the protein that activates proheparanase, CTSL.[238, 259-262] Thus, in addition to

transcriptional changes in FH, it was hypothesised that there may be transcriptional changes in other molecules that affect FH such as FHR proteins, neuraminidases and proteins that mediate HS turnover in circulating cells during dengue.

Publicly available datasets compatible with the R graphical user interface, GEO2R (NCBI), are invaluable and have been used in over 20,000 published manuscripts.[263] Much can be revealed for any disease with a gene expression omnibus series (GSE) dataset without requiring repeated experiments or the data processing resources needed for new microarray projects.[263] These datasets also allow for detailed characterisation of patient groups through repeated analysis by the scientific community. Through analysis of existing, publicly available microarray data, levels of mRNA for FH, FB, FHR-1-5 as well as HS and sialic acid-interacting proteins (HGSNAT, HPSE, HSPG2, CTSL and NEU1-4) in dengue patients were investigated. The mRNA for these proteins in recently presenting (<3 days post symptom onset) dengue patient whole blood samples was compared with dengue PCR-negative febrile disease controls, severe dengue patients and patient-matched convalescent dengue samples.[92] One dataset also allowed for the comparison of primary dengue patients to secondary dengue.

III.2 Results

III.2.1 AP related transcripts are higher during dengue than other febrile illnesses

GSE datasets compatible with GEO2R containing microarray data for dengue patients were identified and studies with sufficient power to return significant results were included in this chapter. The GEO dataset GSE28405 **(Table III-1)** was used to assess mRNA abundance for genes of interest in dengue patients relative to two control types. Samples were collected 1-3, 5-7 and 21-28 days after first symptoms and referred to by the authors as “acute phase, defervescence and convalescence” respectively. The first control group consisted of acute phase patient blood from individuals with febrile illness but were DENV-negative by PCR. The second group of controls came from samples taken during dengue patient convalescence, in theory collected long enough after infection that they reflect basal mRNA abundance of the same patients. GSE28405 was selected due to its high power (n=57) and inclusion of 2 controls. These data were analysed using GEO2R. *RSAD2*, which encodes the potent antiviral gene regulator viperin, was used as an internal mRNA control as it is known to increase in response to flaviviruses such as DENV[264, 265] but also unrelated viruses such as influenza A virus and human cytomegalovirus amongst others.[266-270]

Comparisons of acute phase dengue patients to acute phase febrile DENV PCR-negative patients were used to understand changes in gene expression unique to dengue. These comparisons revealed viperin, FB and FH mRNA were higher for dengue patients during acute disease (4, 12 and 3.8 fold higher respectively) relative to patients with non-DENV acute febrile illness **(Figure III-1A)**. The results in **Figure III-1A** were used in the publication Carr *et al.,* (2020)[271] Figure 4A. Comparison of patient samples during acute disease compared to convalescence were used to assess changes in mRNA abundance during dengue relative to homeostatic levels of the cohort. Acute dengue samples revealed a large increase in mRNA for FB (141-fold increase) as well as upregulation of viperin and FH (8.5-fold and 3.1-fold respectively) relative to the baseline level of these mRNA’s during convalescence **(Figure III-1B)**.

For FHR proteins, FHR-1 and FHR-5 mRNA were approximately half as abundant in dengue patients compared to dengue-negative febrile controls, independent of disease phase. FHR-2 was acutely downregulated (1.25-fold lower), increasing during

defervescence and convalescence (1.1 and 1.9-fold respectively) relative to dengue-negative controls. FHR-3 mRNA was increased during the acute and defervescence phases of disease (2.4 and 2.7-fold respectively) relative to dengue-negative febrile controls and were not significantly different from febrile controls by convalescence. Acute phase dengue patient FHR-4 mRNA was not different from dengue-negative febrile controls **(Figure III-1C)**. No significant difference in the abundance of mRNA for FHR-1, FHR-4 or FHR-5 between acute and convalescent dengue patient samples was observed. However, FHR-2 mRNA was decreased (2.5-fold lower), and FHR-3 mRNA increased (2.4-fold higher) in acute dengue samples when compared to mRNA from patient matched convalescent samples **(Figure III-1D)**.

The fold change in FH and FB relative to convalescence for acute and defervescent disease phases in dengue and dengue-negative patients were expressed as a ratio of FH increase:FB increase. Acutely, FB mRNA demonstrated greater increases in relative abundance than FH (0.08:1 and 0.29:1 for FH:FB in acute non-dengue and dengue febrile illness respectively). However, by defervescence, the increase in FH mRNA is equivocal to the increase in FB mRNA (1:1.15, FH:FB). During convalescence, the ratio of FH mRNA to FB mRNA abundance greatly favours FH (12.5:1 FH:FB). **(Figure III-1E)**.

Table III-1 The GEO datasets containing RNA data for dengue patients from whole blood samples. Detailed information for each sample is accessible through us of the GEO accession.

GEO Dataset (Accession)	Patient Cohort	Country	Control Type	Patient Age range (years)	Time of collection	Number of Healthy Controls (No Treatment)	Number of Dengue Patients (No Treatment)	Number of Severe Dengue Patients (No Treatment)	Reference	Reason for Exclusion
GSE25001	Acute dengue, Severe dengue	Vietnam	No healthy or convalescent controls	2-29	Less than 72 after first symptoms	0	56	24	[272]	**Not Excluded**
GSE28405	Acute dengue, Acute Dengue-negative febrile illness	Singapore	Patient-matched convalescent samples	21-67	<3, 7 and >14 days after first symptoms	26	31	0	[92]	**Not Excluded**
GSE28988	Acute dengue primary vs secondary infection	Singapore	Patient-matched convalescent samples	23-66	<3, 3-7, and >14 days after first symptoms	53	53	0	Submitted to NCBI but not published	**Not Excluded**
GSE40165	Prednisolone/Placebo treated children and adolescents with dengue	Vietnam	Patient-matched convalescent samples	5-20	Less than 72 hours post infection and 1 month after illness subsided	40	40	0	[273]	**Not Excluded**
GSE43777	Acute dengue, Severe dengue	Venezuela	Patient-matched convalescent samples	5-32	Daily for 6 days	13	13	13	[274]	Did not include probes for *RSAD2* or *CFH*

GEO Dataset	Patient Cohort	Country	Control Type	Patient Age range (years)	Time of collection	Number of Healthy Controls (No Treatment)	Number of Dengue Patients (No Treatment)	Number of Severe Dengue Patients (No Treatment)	Reference	Reason for Exclusion
GSE13052	Children with severe or uncomplicated dengue	Vietnam	Patient-matched convalescent samples	9-14	4 days after symptom onset	6	9	9	[275]	Insufficient power
GSE51808	Uncomplicated dengue	Thailand	Yes	0-50	Day 2 after symptom onset and >4 weeks after symptoms	9	18	6	[276]	Insufficient power
GSE96656	Seronegative DENV-3 infected children	Nicaragua	Healthy Controls	0.5-14	"Acute primary infection"	9	24	7	[277]	No probes for CFH, *CFHR1-5*, NEU1-3 or HSPG2

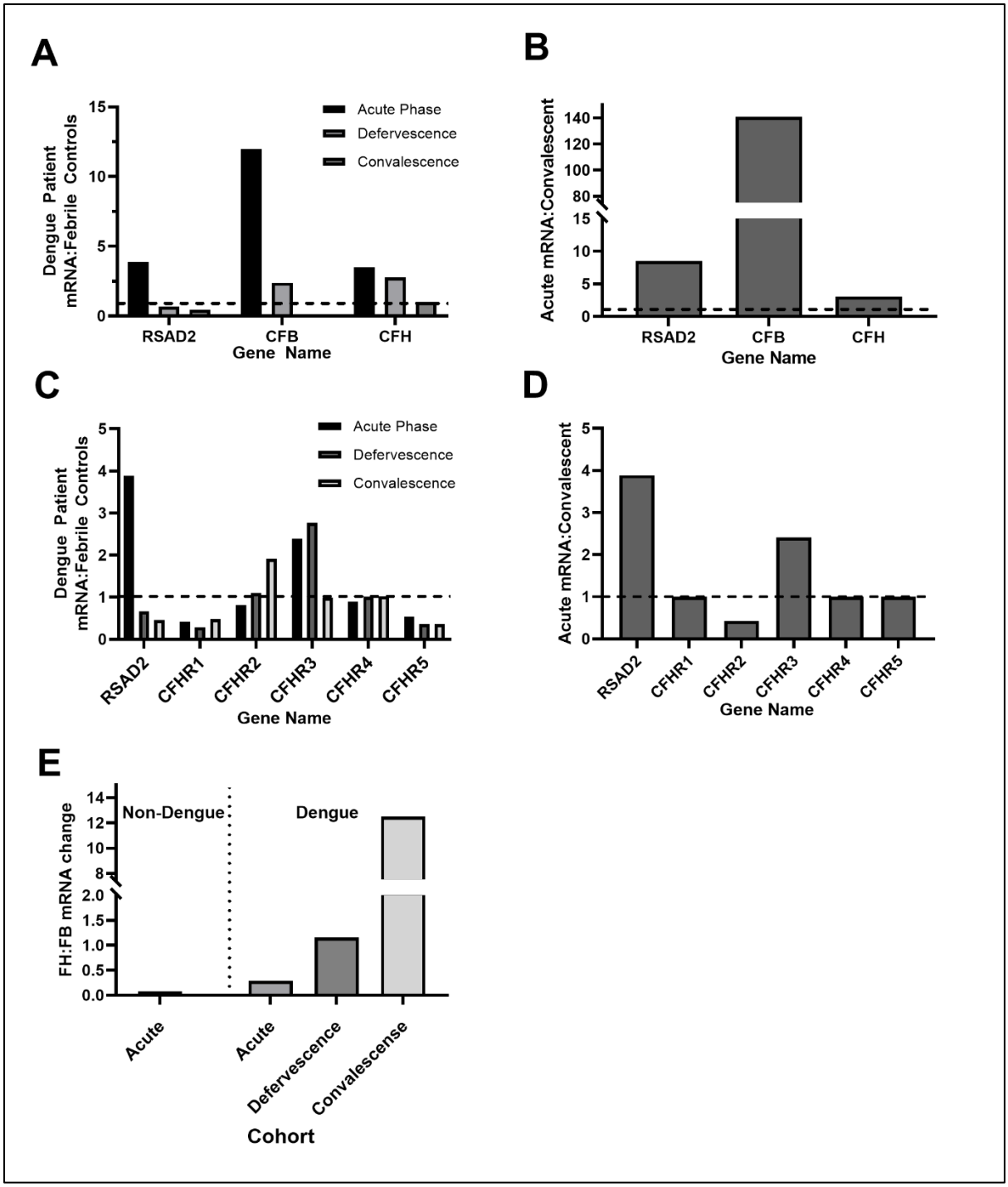

Figure III-1. Dengue acutely increases AP proteins greater than non-dengue febrile illnesses

GEO2R analysed microarray data of GSE28405 comparing relative abundance of AP family mRNAs using **(A+C)** samples collected during acute disease, defervescence and convalescence relative to acutely febrile DENV PCR-negative patients and **(B+D)** patient matched samples of acute disease compared to convalescence. **(A+B)** Relative abundance of viperin, factor B, factor H and **(C+D)** the factor H related protein transcripts in acute dengue patients. All “non-1” values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA from **(A+C)** febrile DENV PCR-negative control blood and **(B+D)** patient-matched blood samples acquired during convalescence. **(E)** The relative difference in change for FH compared to FB during acute dengue-negative febrile illness and across three phases of dengue.

III.2.2 FB mRNA but not FH or FHR1-5 was increased for a Vietnamese cohort with dengue

Abundance of FH, FB, and FHR-1-5 mRNA, accompanied with viperin controls were compared between acute and convalescent whole blood RNA in children with dengue from the GEO dataset GSE40165 using the same method from **Section III.2.1**. Controls for this experiment were patient-matched convalescent whole blood (28 days after last fever day). This study recruited dengue patients 5-20 years old receiving placebo, low-dose, or high-dose prednisolone and only samples from placebo-treated patients (acute and convalescent) were utilised for this analysis. During acute phase disease, defined by the researchers as 0-3 days after first fever day, a significant increase in viperin (15.1-fold) and FB (2.6-fold) mRNA was observed but no significant difference was detected for FH or FHR1-5 mRNA **(Figure III.2)**.

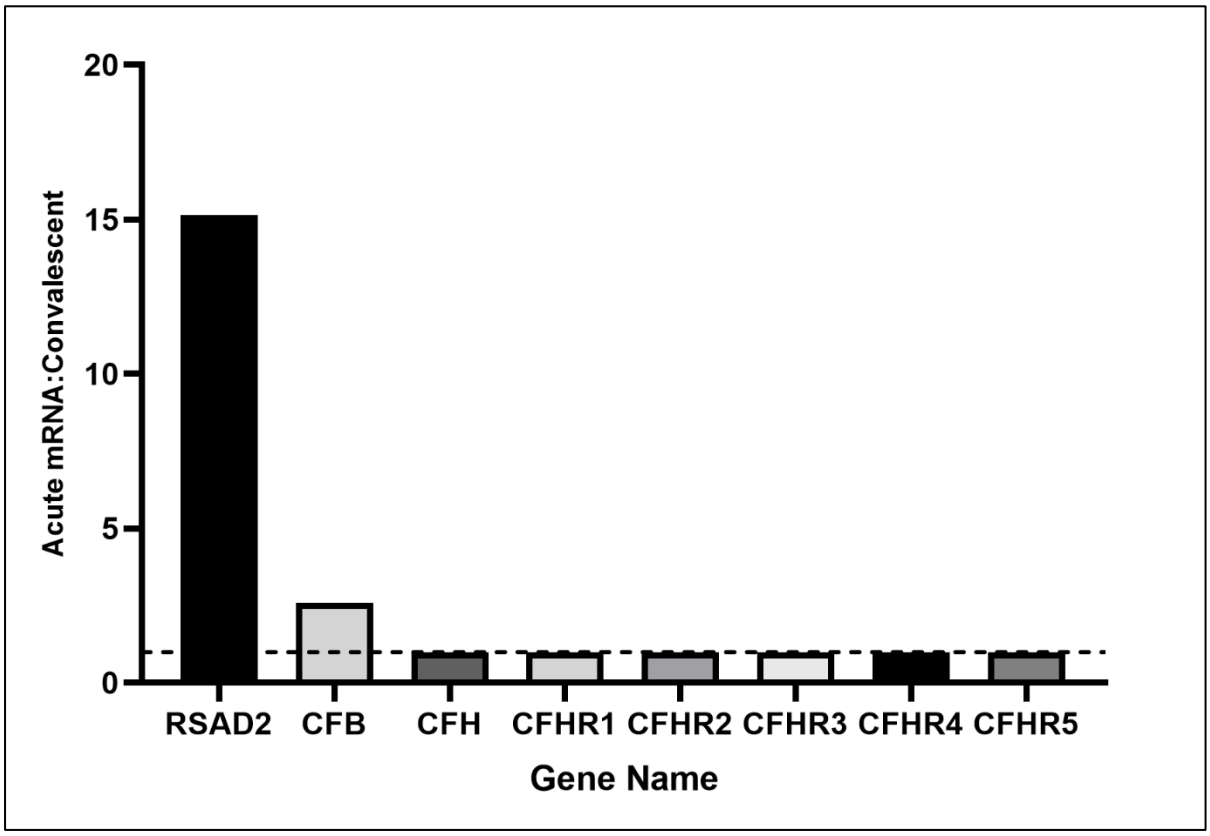

Figure III-2 FB but not FH or FHR1-5 is upregulated during acute phase dengue in a cohort of Vietnamese children.

GEO2R analysed microarray data of GSE40165 comparing relative abundance of alternative pathway family mRNAs using samples collected during acute disease and convalescence. All “non-1” values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA from patient-matched blood samples acquired during convalescence.

III.2.3 Primary dengue produces higher FB than secondary dengue but not FH

Abundance of FH, FB, and FHR-1-5 mRNA, accompanied with viperin controls were compared between acute and convalescent whole blood RNA for the GEO dataset GSE28988 using the same method from **Section III.2.1**. GSE28988 was selected due to sufficient power and inclusion of one control **(Table III-1)**. Control types available for this study were patient-matched convalescent whole blood for both primary and secondary dengue patients, as in GSE28405. Dengue patient, but not severe dengue patient data, were available in GSE28988. For GSE28988, patients were confirmed DENV-positive by NS1 ELISA and anti-DENV IgM and then stratified based on anti-DENV IgG serostatus (i.e. primary and secondary infection) before 3 days post-symptom onset. The IgG serostatus was not further investigated to also include the serotype patient IgG was raised against.

Primary dengue patients had 15 and 17-fold change increases in viperin and FB mRNA respectively, accompanied by a smaller but significant increase in FH mRNA (2-fold) during acute dengue compared to convalescent samples **(Figure III-3A)**. In secondary dengue patients, increased mRNA for viperin (22.2-fold), and FH (2.7-fold) were also observed but FB was not as highly elevated as in primary infection (7.8-fold increased) when comparing acute and convalescent patient matched samples. These differing FH and FB expression profiles may reflect lessened AP activity in secondary infections relative to primary. **(Figure III-3B)**. When primary and secondary infection patient data were combined, comparison of acute and convalescent mRNA for viperin, FB and FH were still significantly increased **(Figure III-3C)**. Direct comparison of primary and secondary dengue revealed that viperin and FH mRNA abundance during acute dengue were not significantly different, thus independent of anti-DENV IgG. However, FB was significantly higher in primary dengue patients relative to secondary infection during acute phase disease (1.7-fold) **(Figure III-3D)**.

In acute primary and secondary dengue patients alike, FHR-1-4 mRNA was 3-fold lower than at convalescence, but FHR-5 mRNA was not within the detectable limits of the assay **(Figure III-4A-B)**. The same result was true for comparison of FHR1-5 mRNA between acute and convalescent disease phases, regardless of whether patients were experiencing a primary or secondary infection **(Figure III-4C)**. FHR-2 mRNA was significantly lower in primary dengue patients relative to secondary dengue

patients during acute disease but there was no significant difference detected between the cohorts for viperin, FHR-1, 3 or 4 mRNA and FHR-5 mRNA remained below the limit of detection. **(Figure III-4D)**.

When comparing mRNA abundance between primary and secondary dengue patients during defervescence (4-7 days post symptom onset), no genes targeted in this chapter were significantly different, including RSAD2 (raw data available at zenodo.org/record/6937603#.YuOrT3ZByUk). Comparison of transcript abundance between patients who experienced a primary or secondary infection at convalescence (after full recovery) returned no significant differences for any gene, as expected (raw data available at zenodo.org/record/6937603#.YuOrT3ZByUk).

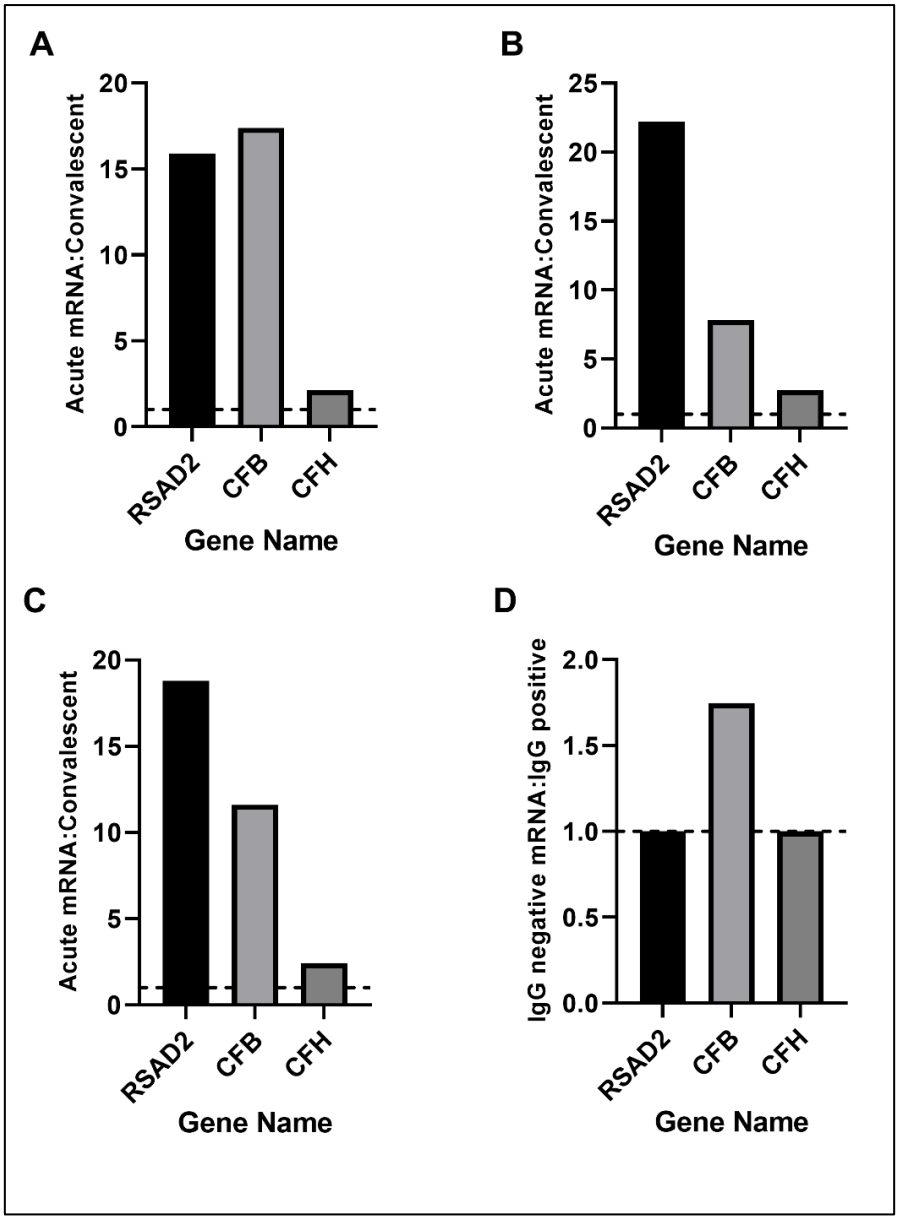

Figure III-3 Primary dengue induces higher FB but not FH mRNA during acute disease.

Dengue patients were stratified by DENV anti-DENV IgG less than 3 days after symptoms onset and had whole blood collected at <3 and >2 weeks post symptom onset. Whole blood underwent microarray analysis, and data were made publicly available as the GEO dataset GSE28988. The abundance of mRNA transcribed from *RSAD2*, *CFB* and *CFH* during the acute phase of disease was compared for each of anti-DENV **(A)** IgG negative and **(B)** IgG positive to corresponding patient-matched convalescent blood. **(C)** Data of combined patient mRNA abundance for the targeted genes, regardless of anti-DENV IgG serostatus, was compared to patient matched convalescent mRNA abundance. **(D)** Patient mRNA abundance for samples collected <3 days post symptom onset were compared between primary and secondary dengue patients. All “non-1” values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA from **(A-C)** patient matched convalescent samples **(D)** DENV IgG positive samples collected <3 days post symptom onset.

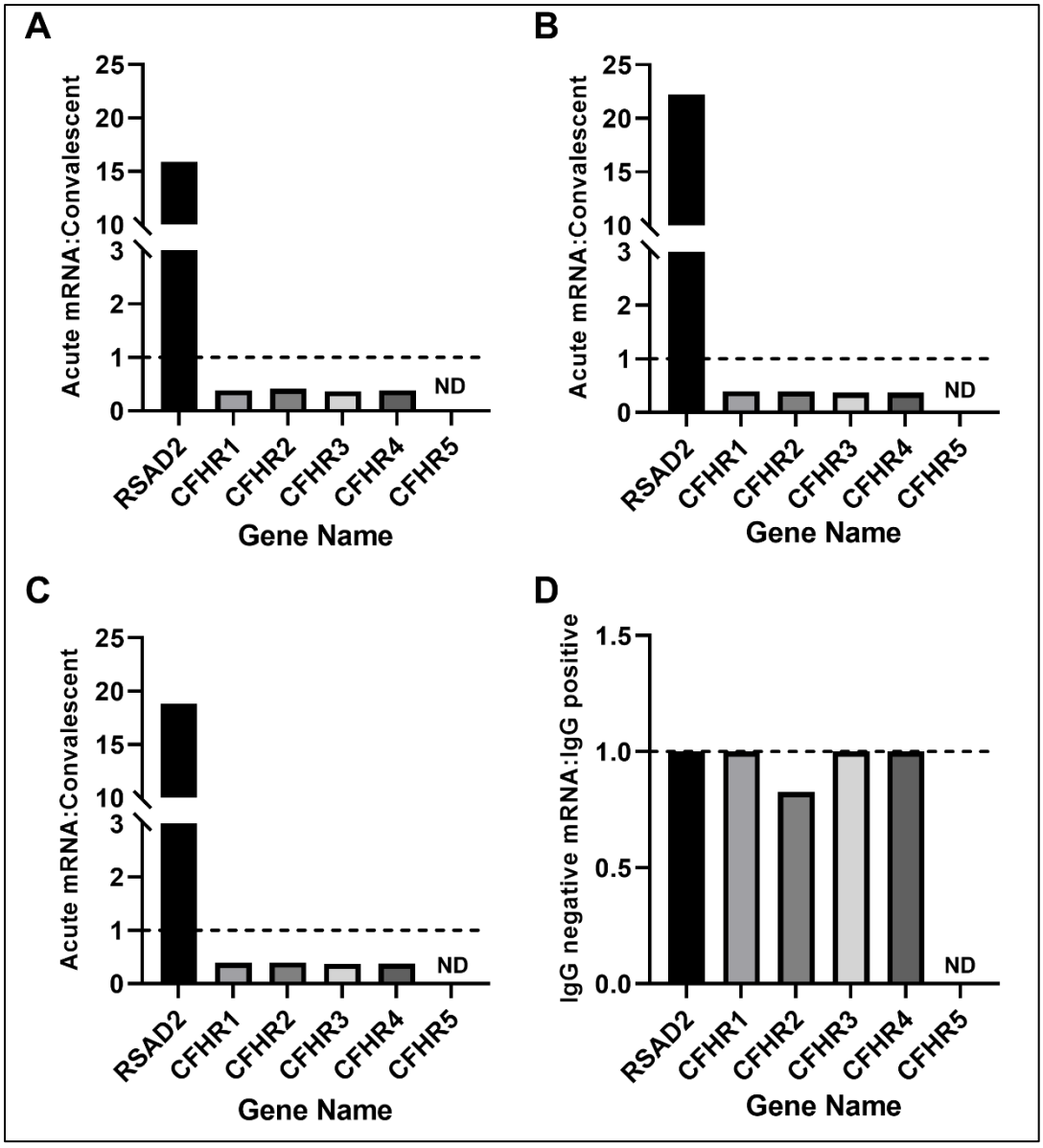

Figure III-4 Dengue causes reduction of mRNA for FHR1-4 but FHR5 mRNA was below the limit of detection in GSE28988.

Dengue patients were stratified by the detection of anti-DENV IgG and had whole blood collected at <3 and >2 weeks post symptom onset. Whole blood underwent microarray analysis, and data were made publicly available as the GEO dataset GSE28988. The abundance of mRNA transcribed from *RSAD2* (encoding viperin) and *CFHR1-5* during the acute phase of disease was compared for each of anti-DENV **(A)** IgG negative and **(B)** IgG positive to patient-matched convalescent blood. **(C)** Data of all patient mRNA abundance for the targeted genes regardless of anti-DENV IgG was also compared to patient matched convalescent mRNA abundance. **(D)** Patient mRNA abundance for samples collected <3 days post symptom onset were compared between primary and secondary dengue patients. All "non-1" values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA from **(A-C)** patient matched convalescent samples or **(D)** DENV IgG positive samples collected <3 days post symptom onset. ND=Not detected

III.2.4 Severe dengue patient blood has increased abundance of FH and FB transcripts

Patient studies have revealed circulating FH is decreased during dengue relative to severe dengue, correlating with increased complement activity, likely due to less regulation.[217] As FH mRNA is apparently increased in response to DENV infection *in vitro* and acutely increased in patients with dengue **(III.2.1-3)** the relative mRNA quantities of FH, FHR-1-5 and FB in dengue and severe dengue patients were compared. GSE25001 was one of four (including GSE43777, GSE13052 and GSE96656) datasets to compare dengue and severe dengue patients but was the only dataset with probes for the genes of interest or sufficiently high power to detect significant changes in mRNA abundance **(Table II-1)**. As in **Section III.2.1**, GEO2R was used to compare mRNA from cells in whole blood taken during the acute phase of dengue, prior to onset of severe disease, and analysis performed, comparing patients that developed severe dengue to those who did not.

Viperin mRNA was not significantly different between dengue and severe dengue patients. FB and FH mRNA abundance were higher in patients that developed severe dengue compared to dengue (2.75 and 1.52-fold increase respectively) **(Figure III-5)**. FHR-1 and FHR-3 mRNA were slightly decreased in patients who developed severe dengue compared to those who did not develop severe dengue (1.2 and 1.05-fold reduction respectively). FHR-2 mRNA was not significantly different between dengue and severe dengue patients. FHR-4 and FHR-5 mRNA abundance were higher (1.35 and 2-fold increase respectively) in severe dengue patients relative to dengue **(Figure III-5)**.

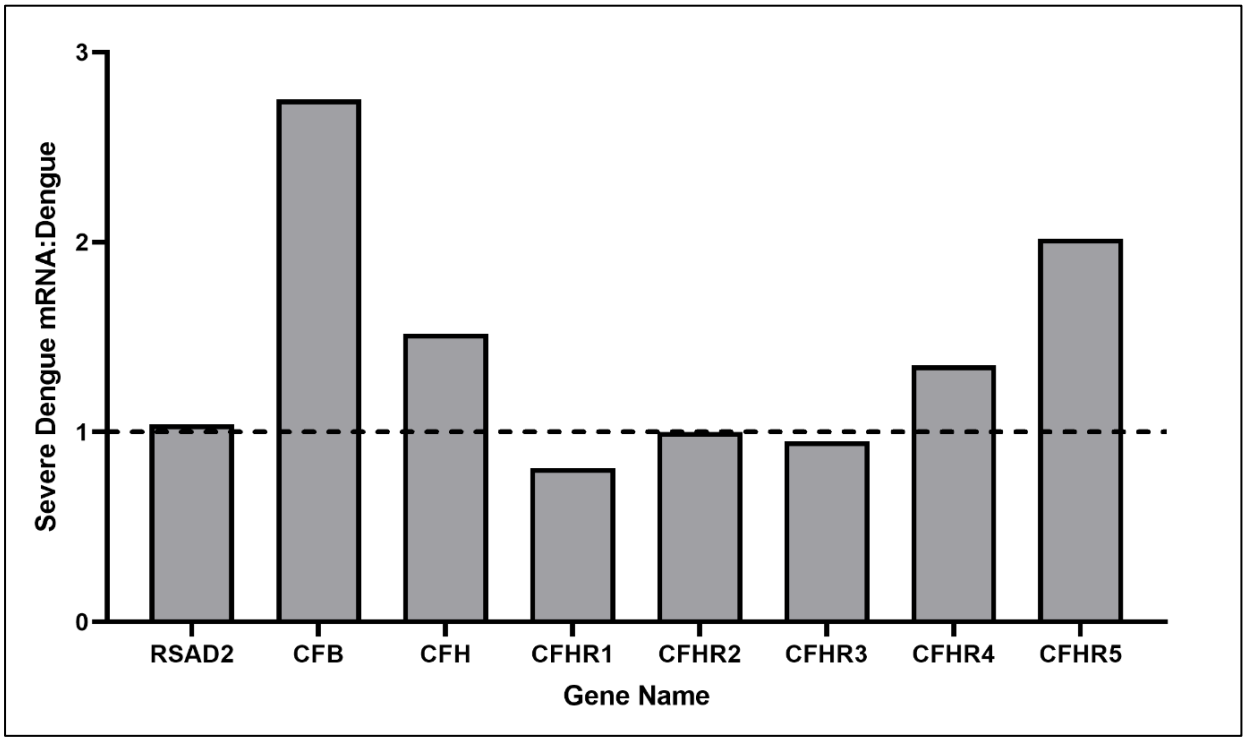

Figure III-5 Severe dengue patients have higher abundance of both alternative pathway activators and regulators.

GEO2R analysed microarray data from GSE25001 for fold-change expression of mRNA transcripts in severe dengue patients from blood taken 3-5 days after first symptoms compared to acute dengue patients from blood at the same timepoint. All “non-1” values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA from dengue patient blood.

III.2.5 CTSL transcripts are highly abundant during DENV infection but not heparanase or neuraminidases

The publicly available microarray data from GSE28405, GSE40165, GSE 28988 and GSE25001 from **Section III.2.1-4** were also used to compare transcripts for the proteins that control FH-ligand interacting molecules. These proteins are important in modulating host-membrane ligands for FH, HS, and sialic acid. As in **Section III.2.1**, when analysing GSE28405, results represent the comparison of the three phases of dengue: acute, defervescence and convalescence. Values are expressed relative to DENV PCR-negative febrile patient mRNA (**Figure III-6A**) and patient-matched comparison of acute and convalescent mRNA in cells collected from whole blood (**Figure III-6B**). GSE25001 was used as in **Section III.2.3** to compare the mRNA abundance between dengue and severe dengue however, returned no significant difference for any of the genes investigated in this chapter.

Heparanase (HPSE) and HPSE-activating protein CTSL were selected due to their combined ability to reduce HS abundance on the plasma membrane. HGSNAT was selected due to its role in lysosomal degradation of HS, reducing HS abundance.[213] HSPG2 was selected as it anchors HS to the cell membrane and is vital in maintaining the glycocalyx and endothelial barrier function, which are disrupted during DENV.[278] The neuraminidase family (NEU1-4) were selected due to their role in sialic acid turnover and also disrupt the glycocalyx.[15]

Significantly lower mRNA abundance was observed for HPSE (0.88, 0.55 and 0.60-fold) and NEU2 (0.6, 0.79 and 0.53-fold) for acute phase, defervescence and convalescence in individuals with dengue relative to dengue-negative febrile patients. HGSNAT and HSPG2 mRNA were higher in all disease phases relative to dengue-negative febrile patients. HGSNAT mRNA increased (1.51, 1.35 and 2.91-fold higher) and HSPG2 mRNA trended towards a decrease (2.85, 2.75 and 1.95-fold higher) in dengue relative to dengue-negative febrile patients over the acute-phase, defervescence and convalescence. CTSL and NEU1 mRNA were significantly higher, approximately 5 and 1.5-fold higher respectively in dengue patients during acute disease relative to dengue-negative febrile patients. NEU4 mRNA demonstrated a slight but significant increase (1.28, 1.13 and 1.51-fold higher) for all the acute phase,

defervescence and convalescence in patients with dengue relative to relative to dengue-negative febrile patients. **(Figure III-6A)**.

Comparing patient-matched acute and convalescent dengue samples, acute dengue caused increased CTSL and NEU1 mRNA (19.8 and 2.1-fold increase respectively) and these were the only significant changes in relative gene abundance, other than viperin, for this cohort. **(Figure III-6B)**.

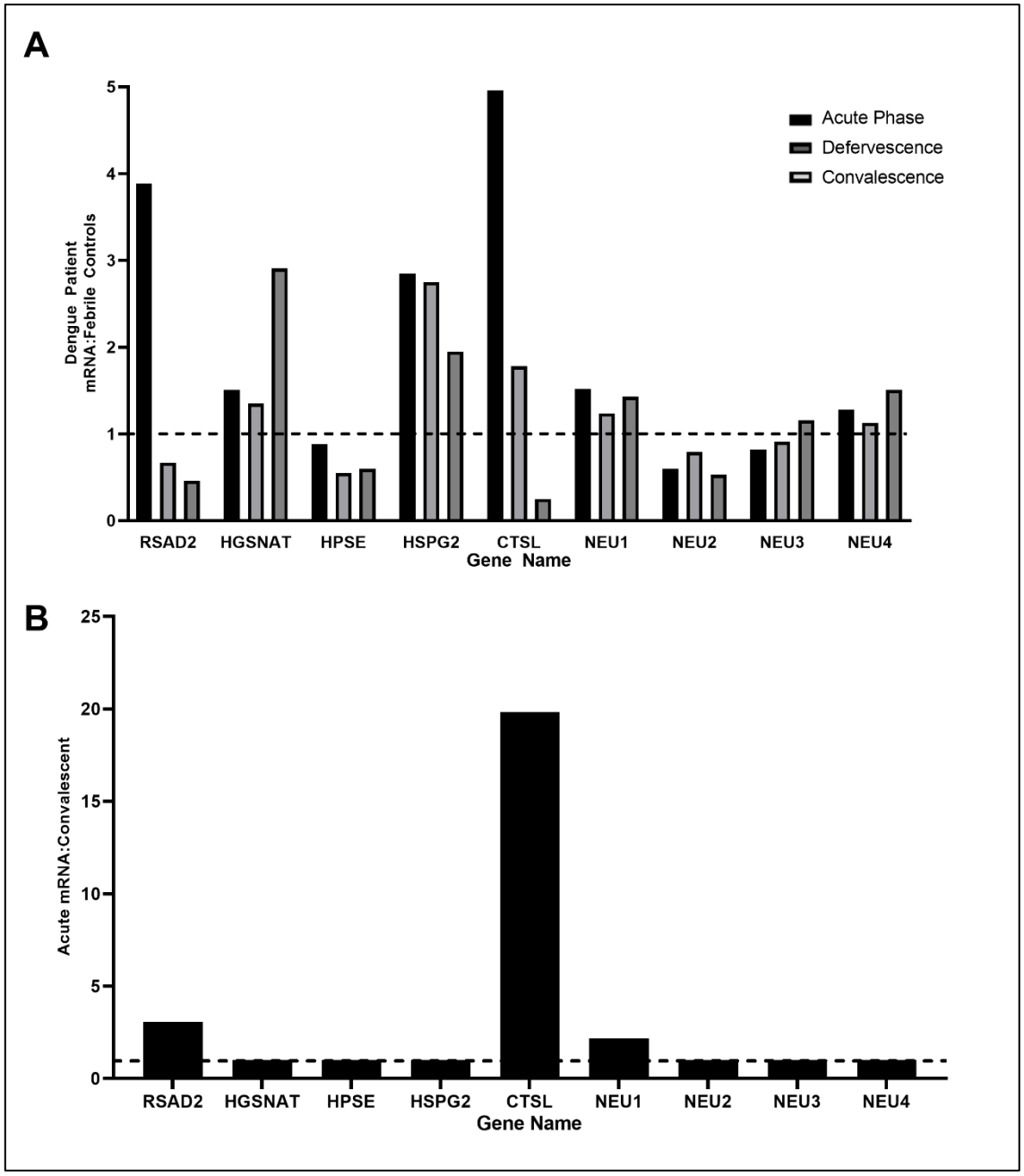

Figure III-6 CTSL mRNA is highly increased during acute DENV infection in whole blood cells.

GEO2R analysed microarray data of GSE28405 comparing relative levels of alternative pathway family mRNA using **(A)** samples collected during acute disease, defervescence and convalescence relative to acutely febrile DENV PCR-negative patients and **(B)** patient matched samples of acute disease compared to convalescence. All "non-1" values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA abundance of (A) PCR-negative, febrile patient blood taken during acute disease (B) patient-matched blood samples acquired during convalescence.

III.2.6 HPSE, CTSL and NEU1 increase during acute dengue in a cohort of Vietnamese children

As in **Section III.2.5**, GSE40165 was used to compare mRNA abundance for HGSNAT, HSPG2, HPSE, CTSL and NEU1-4 to define changes in FH-ligand interacting enzymes, specifically those that interact with HS and sialic acid. Comparative analysis was performed for mRNA abundance in dengue patients aged 5-20 between acute (0-3 days after fever start) and convalescent (>28 days after last fever day) phases of diseases, using samples of whole blood.

HPSE, CTSL and NEU1 were each significantly increased (3, 7 and 2.2-fold respectively) and NEU3 was significantly lower (1.16-fold decrease) during dengue relative to patient mRNA abundance during convalescence. HSPG2, NEU2 and NEU4 were not significantly different **(Figure III-7)**.

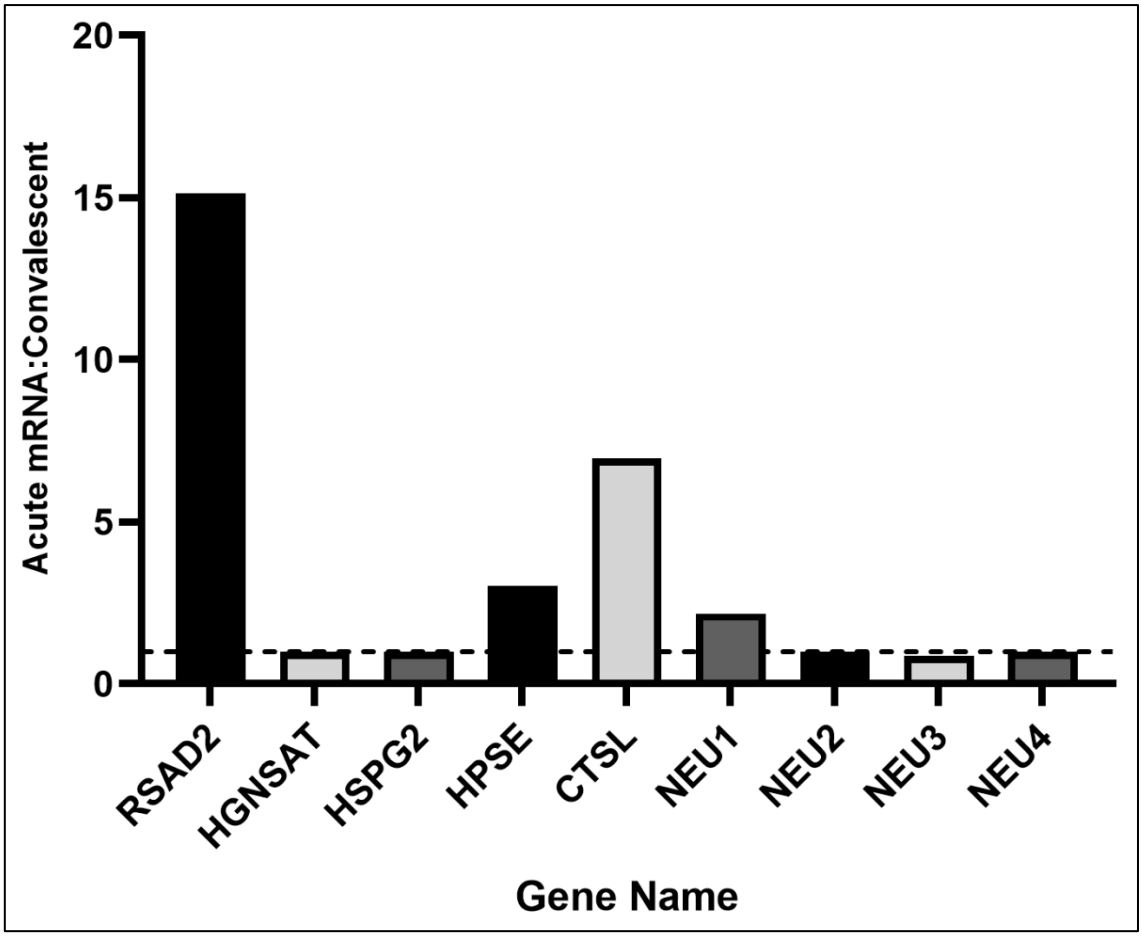

Figure III-7 HS degrading enzymes are upregulated during acute phase dengue in a Vietnamese dengue patient cohort

GEO2R analysed microarray data of GSE40165 comparing relative abundance of HS (HGSNAT, HSPG2, HPSE, CTSL) and sialic acid (NEU1-4) interacting enzyme mRNAs using samples collected during acute disease and convalescence. All “non-1” values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA from patient-matched blood samples acquired during convalescence.

III.2.7 mRNA for HS interacting proteins increase but not for neuraminidases during DENV infection, independent of anti-DENV IgG

As in **Section III.2.5-6**, GSE28988 was used to compare the abundance of mRNA between acute and convalescent whole blood samples, comparing primary and secondary infection, analysing FH-ligand interacting proteins. In primary and secondary dengue patients, mRNA for the following genes were significantly increased during the acute phase of disease relative to patient matched convalescent samples: HPSE (3.6-fold for both), HSPG2 (3.5 and 2.2-fold), CTSL (6.3 and 3.9-fold), NEU1 (2.1 and 1.8-fold) for primary and secondary dengue patients, respectively. NEU2 was significantly decreased (3 and 2.8-fold, primary and secondary dengue respectively) during acute disease phase relative to convalescence. Additionally in secondary dengue patients decreased NEU4 mRNA (1.6-fold lower) was observed **(Figure III-8A-B)**. When combined, the same relative changes described above for IgG positive and IgG negative groupings were observed, however, the NEU4 mRNA decrease previously observed in the secondary dengue group was no longer significant **(Figure III-8C)**. A small but significant increase in the abundance of mRNA for NEU4 was detected in primary dengue patients compared to secondary dengue (1.27-fold increase) during acute phase disease. Apart from NEU4, there was no apparent difference in the levels of HS and sialic acid-interacting proteins targeted by these analyses between primary and secondary DENV infected patients. as none of the mRNA abundances for the targeted proteins was significantly different **(Figure III-8D)**. No significant differences between primary and secondary dengue patient mRNA abundance were observed for samples collected at 3-7 days, or at 28 days post infection for the transcripts analysed in this section as reported in **Section III.2.3**. Results for **Section III.2.1-7** are summarised in **Table III-2.**

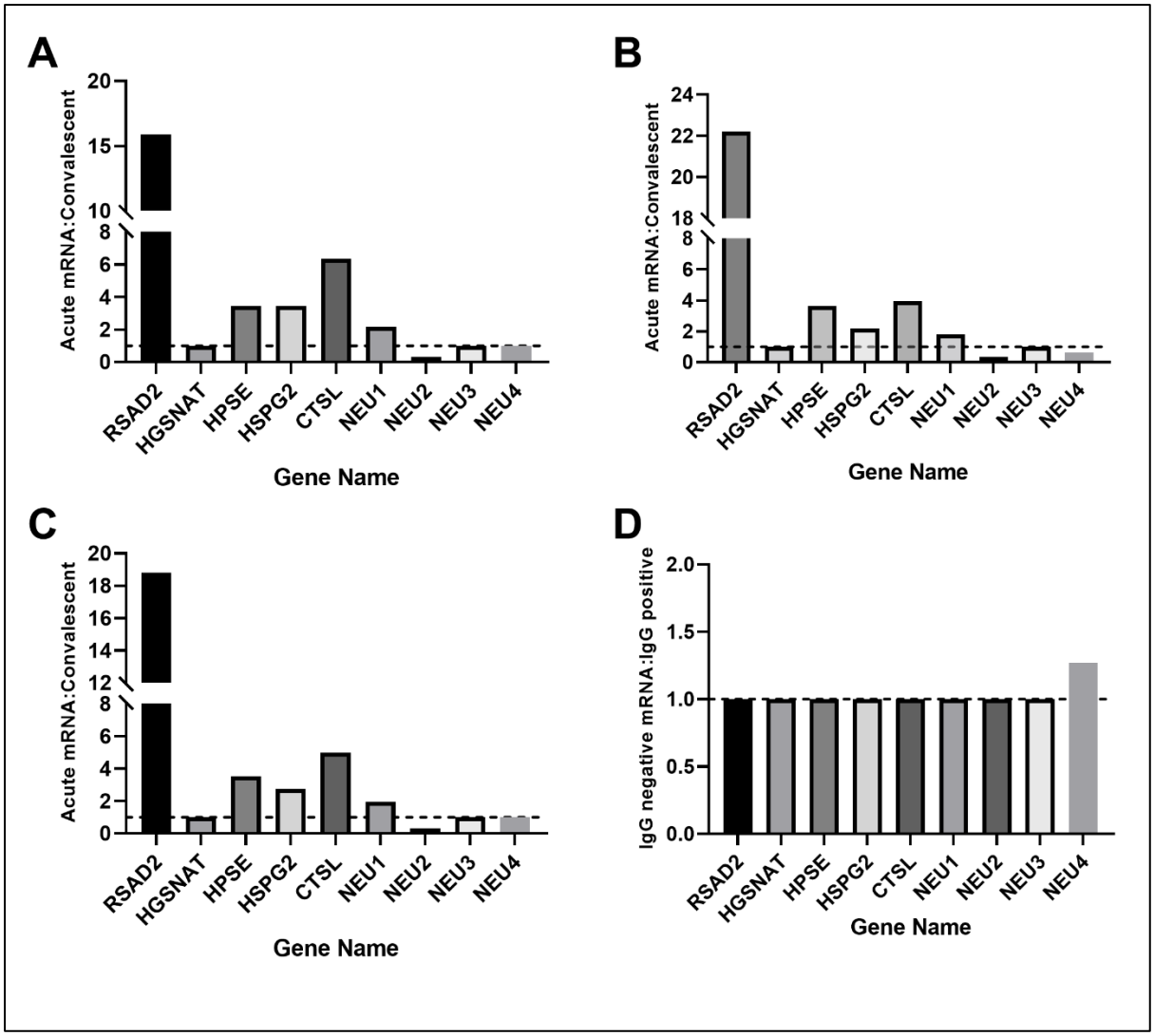

Figure III-8 HS and sialic acid interacting proteins are increased during dengue but only NEU4 mRNA is significantly different between primary and secondary infection.

Dengue patients were stratified by the presence of anti-DENV IgG and had whole blood collected at <3, 3-7 and >2 weeks post symptom onset. Whole blood underwent microarray analysis, and data were made publicly available as the GEO dataset GSE28988. The abundance of mRNA transcribed from RSAD2 (encoding viperin), HGSNAT, HPSE, HSPG2, CTSL and NEU1-4 during the acute phase of disease was compared for each **(A)** primary dengue and **(B)** secondary dengue to patient-matched convalescent blood. **(C)** Data of all patient mRNA abundance for the targeted genes regardless of patient's previous exposure to DENV was also compared to patient matched convalescent mRNA abundance. **(D)** Patient mRNA abundance for samples collected <3 days post symptom onset were compared between primary and secondary dengue patients. All "non-1" values shown were determined to be significant by GEO2R using p-values <0.05 after Benjamini & Hochberg adjustments. Dashed line at "Fold change = 1" represents mRNA from **(A-C)** patient matched convalescent samples **(D)** DENV IgG positive samples collected <3 days post symptom onset.

Table III-2 Summary of transcript abundance during acute disease for complement proteins and proteins that interact with HS and sialic acid during dengue.

A summary of the results for **Sections III.2.1-7** displaying changes in abundance for mRNA of two gene panels during dengue, as determined using existing microarray data. Changes during dengue are increased or decreased if at least two of the three studies demonstrated this pattern and the results of all three studies were not in conflict. Change during severe dengue were determined using one publicly available microarray dataset. No gene transcribed for heparan sulphate and sialic acid interacting proteins was different during severe dengue and therefore are not displayed. Red represents genes increased during illness, blue were decreased, grey are unchanged (none) and white represents genes with results that conflicted between studies (undetermined).

Genes Transcribed for RSAD2 and Complement Proteins								
Gene Name	*RSAD2*	*CFB*	*CFH*	*CFHR1*	*CFHR2*	*CFHR3*	*CFHR4*	*CFHR5*
Change during dengue	Increased	Increased	Increased	Undetermined	Decreased	Undetermined	None	None
Change during severe dengue	None	Increased	Increased	Decreased	None	Decreased	Increased	Increased
Genes Transcribed for Heparan Sulphate and Sialic Acid Interacting Proteins								
Gene Name	*HGSNAT*	*HPSE*	*HSPG2*	*CTSL*	*NEU1*	*NEU2*	*NEU3*	*NEU4*
Change during dengue	Undetermined	None	Increased	Increased	Increased	None	None	None

III.2.8 The *CFH* gene contains more predicted transcription factor binding sites than CFHRs and uniquely possesses a binding site for the NF-kB subunit 'RelA'

The *CFHR* genes arose from duplications of the *CFH* parent gene and are unique to higher order primates, although other mammals have *CFHR*-like genes that have convergently evolved.[253] It is now understood that the FHR proteins broadly interact with the complement AP without performing many of the roles of the parent FH molecule.[251] Due to the observations in **Sections III.2.1-4** that FH and FHRs have disparate patterns of mRNA abundance following infection, the 1200 nucleotides upstream of the transcription start site for *CFH* and *CFHR1-5* were analysed by the promoter analysis online tool PROMO (Alggen). By chance, short consensus sequences occur more frequently than long consensus sequences. Therefore, an estimate of how many sites predicted to occur by chance were also included in the right most column to serve as a control.

There were 5 development-associated TFs detected, which were predicted to be present in all 6 analysed promoter regions. At least 2 liver-associated TFs were present in each of the 6 *CFH* and *CFHR* promoter regions analysed and *CFHR1* was predicted to contain all 5 of those detected (C/ebpα, HNF-1A, HNF-1B, HNF-3α, HNF-4α). There were 15 immune-associated TFs predicted to bind the *CFH* and *CFHR1-5* promoter regions. Of interest was the prediction of a RelA binding site unique to the *CFH* promoter region.

CFHR1 had the lowest number of predicted immune-associated TF binding elements. POU2F2 binding sites were identified in *CFHR1* and *CFHR5* only. Despite being present in most of the other genes analysed, *CFHR1* was not predicted to have binding elements for ERα, PAX-5 or PXR-1:RXR-A. *CFHR1* was predicted to have binding elements for DNA damage and apoptosis-associated factors ATF-3 and Sp1. *CFHR2*, was predicted to lack the PXR-1:RXR-A element present in the *CFH* and *CFHR3-5* promoters.

CFHR2 was also one of the two genes analysed with a predicted STAT1β binding element. The *CFHR3* promoter had the lowest number of predicted TF binding elements, with the same as *CFHR1* (11 sites). *CFHR3* lacked IRF-1 and IRF-2 predicted sites which were present in most other *CFHR* promoters in some combination. The *CFHR4* promoter was predicted to contain most of the TF binding

elements present for the other members of the *CFHR* family, except for IRF-1, which was not predicted. The *CFHR4* promoter was the only gene to have TF binding elements for the apoptosis associated TF, MITF. *CFHR5* contained all immune-associated binding elements also detected in *CFHR1-4*. *CFH*, *CFHR4* and *CFHR5* were the only genes predicted to contain a c-Myb binding site **(Table III-3)**.

Table III-3 The transcription factor binding elements predicted -1100 to +100 bp relative to the *CFH* and *CFHR1-5* transcription start sites.

TF binding sites predicted to occur by chance determined by: $\frac{1}{4^{site\ length}} \times \mathbf{1200}$. Black squares with "X" represent the promoters that did not contain the consensus sequence for a given transcription factor. Genes with a predicted site for the given transcription factor are white with a tick. Genes were separated into columns based on mRNA abundance changes observed in **sections III.2.1**. No site was predicted more than once per promoter region.

	Transcription factor	Increased during dengue or severe dengue			Unchanged during dengue		Decreased during dengue	Number of binding sites predicted to occur by chance
		CFH	*CFHR4*	*CFHR5*	*CFHR3*	*CFHR1*	*CFHR2*	
Developmental								
	AP2α	✓	✓	✓	✓	✓	✓	0.3
	HOXD10	✓	✓	✓	✓	✓	✓	0
	HOXD9	✓	✓	✓	✓	✓	✓	0
	SRY	✓	✓	✓	✓	✓	✓	0
	YY1	✓	✓	✓	✓	✓	✓	0
Hepatic								
	C/ebpα	✓	✓	✓	✓	✓	✓	0
	HNF-1A	✓	X	✓	X	✓	✓	0
	HNF-1B	X	X	✓	X	✓	✓	0
	HNF-3α	✓	✓	✓	✓	✓	✓	0
	HNF-4α	✓	X	X	X	✓	X	0
Immunity								
	C/ebpβ	✓	✓	✓	✓	✓	✓	1.2
	c-Myb	✓	✓	✓	X	X	X	1.2
	Elk-1	✓	✓	✓	✓	✓	✓	0
	ERα	✓	✓	✓	✓	X	✓	0
	FOXP3	✓	✓	✓	✓	✓	✓	0.6
	IRF1	✓		✓	X	✓	✓	0
	IRF2		✓	✓	X	✓	✓	0
	Pax-5	✓	✓	✓	✓		✓	0
	POU2F2			✓		✓		0
	PXR-1:RXR-A	✓	✓	✓	✓			0.6
	RAR-β	✓	✓	✓	✓	✓	✓	0.3
	RelA	✓						0
	RXR-α	✓	✓	✓	✓	✓	✓	0
	STAT1β			✓			✓	0
	STAT4	✓	✓	✓	✓	✓	✓	0
DNA Damage/Apoptosis								
	ATF-2	✓						0
	ATF-3	✓				✓		0

	Transcription Factor	Increased during dengue			Unchanged during Dengue		Decreased during dengue	Number of Binding Sites Predicted to Occur by Chance
		CFH	*CFHR4*	*CFHR5*	*CFHR3*	*CFHR1*	*CFHR2*	
DNA Damage/Apoptosis								
	MITF		✓					0
	Sp1					✓		0
	XBP-1		✓	✓	✓	✓	✓	0
Steroidal/Hormonal Response								
	AR	✓						0
	c-Jun	✓						0.1
Miscellaneous								
	c-Ets-2	✓	✓	✓	✓		✓	9.4
	CREB	✓	✓	✓				0
	GATA-2		✓					0.3
	GATA-1	✓				✓	✓	1.2
	GR	✓	✓	✓	✓	✓	✓	0
	GR-A	✓	✓	✓	✓	✓	✓	0
	Ik-1			✓			✓	4.7
	LEF-1	✓	✓	✓	✓	✓	✓	0.1
	NF-1	✓	✓	✓		✓		1.2
	NF-AT1	✓	✓	✓	✓	✓	✓	1.2
	NF-AT2	✓	✓	✓		✓	✓	0
	NFI/CTF			✓				0
	NF-Y	✓	✓	✓		✓	✓	0
	p53	✓	✓	✓	✓	✓	✓	0
	PEA3		✓	✓	✓	✓		0.3
	PR A	✓	✓	✓	✓	✓	✓	0
	T3R-β1	✓	✓		✓	✓	✓	0
	TBP	✓		✓		✓	✓	0
	TCF-4		✓	✓		✓	✓	4.7
	TCF-4E	✓	✓	✓	✓	✓	✓	4.7
	TFIID	✓	✓	✓	✓	✓	✓	0.1
	VDR	✓		✓			✓	0

III.3 Discussion

The results of this chapter defined mRNA abundance from dengue patient blood cells for FH, FB, FHRs, and FH-ligand interacting proteins in response to dengue *in silico*. This was also extended to the patterns of mRNA abundance for these proteins with respect to primary compared to secondary infection and disease severity. Only circulating FH protein but not mRNA had been measured for dengue patients in the past and so, comparing FH mRNA across dengue phases, primary compared to secondary infection and disease severities was needed. The comparisons made within these datasets captured 4 different perspectives on gene expression during dengue in whole blood cells. These comparisons were: relative transcript abundance in acute dengue compared to homeostasis (GSE28405, GSE40165 and GSE28988), the changes in transcript abundance specific to dengue, relative to other febrile illnesses (GSE28405), the differences in mRNA abundance in response to dengue for primary compared to secondary infection (GSE28988) and differences in transcript abundance during the acute phase of disease between dengue and severe dengue (GSE25001).

There are 8 gene expression omnibus (GEO) microarray datasets that exist for dengue patient RNA from whole blood. Of these, GSE28405, GSE40165, GSE28988 and GSE25001 met the criteria for study inclusion. The remaining 4 studies were excluded due to insufficient power and a microarray that did not include probes to detect genes of interest **(Table III-1)**. Thus, the conclusions drawn by this research are derived from the observations made in 4 (three with dengue, one with dengue and severe dengue) cohorts.

The data from mining existing dengue patient microarray GSE datasets (GSE28405, GSE40165 and GSE28988), analysed by GEO2R demonstrated higher abundance of mRNA for viperin during acute phase dengue relative to patient matched convalescent samples. Viperin is known to be induced during dengue and other viral infection and indicates that type I IFN responses were likely activated.[264, 265, 267] With the observation that viperin was induced by dengue as expected, relative mRNA abundance for the proteins that exist in the FH interactome were investigated.

FH is the most important regulator of the AP and its roles in regulation cannot be completely compensated by other proteins.[197, 208, 279, 280] In response to dengue, FH

mRNA was increased acutely in two of three studies, persisting until at least 7 days after symptom onset, was independent of primary compared to secondary dengue and was increased further in severe dengue relative to dengue. In GSE40165, FH mRNA induction was not observed. This could be due to an inherent difference in sample collection timing, as FB mRNA was also not increased to the same magnitude (2.6-fold increase) in GSE40165 as other studies (15 to 120-fold increaseGSE28988 and GSE28405). Increased FH mRNA in response to dengue is in agreement with observations in MDM and EC *in vitro*.[44] This demonstrates that decreased FH protein in severe dengue patients observed by Nascimento et al., (2014)[225] was likely not from decreased FH mRNA by cells in whole blood. However, data from these microanalyses is limited to mRNA only and does not have accompanying quantitation of extracellular FH protein. Instead, post-transcriptional events such as increased FH-cell binding in response to DENV, as seen by Cabezas et al., (2018)[44] for MDM *in vitro*, mRNA degradation or changes in hepatic FH production now appear to be more likely explanations and are explored further in **Chapter IV**.

The proportion of DENV infected cells is low for immune cells (3-12%), with the exception of B-cells (20-80%), derived from whole blood infected *ex vivo* or in dengue patient peripheral blood cells as determined by flow cytometry.[281, 282 283, 284] Potentially, increased FH mRNA is in response to stimuli produced by DENV infected cells rather than the virus itself which is investigated in **Chapter V**. In healthy individuals, FH (150 kDa) and FB (93 kDa) are estimated to circulate at 500 µg/mL[197] and 30 ng/mL[285] respectively (approximately 1000:1 FH:FB as a molar ratio). Dengue also caused a large and acute FB mRNA increase which was higher in patients with severe disease. Increased FB mRNA, which was consistently higher than the relative increases observed in FH mRNA for all 4 studies, is a reflection of the imbalance in regulation and activation that has been repeatedly observed for AP proteins during dengue.[43, 44, 182, 217, 271] Although, the ratio of FH:FB was even lower for dengue-negative febrile patients. This suggests other factors are important for AP dysregulation during dengue than the ratio of FH:FB alone. DENV PCR-negative febrile controls from GSE28405 were valuable in demonstrating that, although febrile disease will induce FH and FB, the magnitude to which dengue increases FH and FB mRNA is higher than in other febrile diseases. The comparison of DENV and dengue-negative febrile illness also

demonstrated that the ratio of increase for FH:FB is not unique to dengue. Potentially, the disparate ratio and magnitude of increased FB to increased FH mRNA during acute disease could mean that the FH protein is unable to maintain complement regulation on healthy cell surfaces. These analyses would have benefited from defervescent sample collection for dengue-negative patients for better understanding of the temporal regulation of FH and FB during dengue relative to other febrile diseases. Utilising GSE datasets for other febrile infectious diseases, such as ZIKV or WNV could help to determine whether changes in AP transcript abundance during dengue exceeds the typical response to a viral infection. Additionally, as with dengue, the amount of AP complement activation in COVID-19 patients has been correlated with disease severity.[286] Specifically, increased FB, decreased C3 and increased C3a all occur for severe COVID-19 patients, reproducing the observations made for dengue patients.[286] Thus, the results from **Chapter III** reveal patterns of transcriptional change for complement proteins during dengue and extension of this methodology to COVID-19 patients may provide insight for both diseases and severe viral infections more broadly. As FH and FB mRNA were both demonstrated to increase in response to dengue, other FH isoforms were of interest due to their role in the AP.

FHR mRNA and protein expression by leukocytes is not well defined and detectable changes in FHR in response to infection by microarray is in itself a novel and interesting finding. For the most part, FHR mRNA abundance in response to dengue did not have clear implications for changes in complement activity in patients. However, the FHR-2 decrease observed for dengue-negative febrile illnesses and also during acute phase dengue demonstrates this is a general response to febrile disease. FHR-2 regulates the AP by reducing C3-convertase activity and its decrease therefore favours AP activation.[197] Furthermore, higher FHR-4 and FHR-5 mRNA for severe dengue patients reinforces the observations that the AP during severe dengue favours activation over regulation, as both FHR-4 and FHR-5 promote C3b binding and thus, AP activity.[197, 206, 256, 287] The proteins that govern AP regulation are influenced by their ability to access sites of AP activation.[186, 237] Additionally, FH specifically cannot regulate membrane bound C3b or the C3-convertase in the absence of sialic acid.[144, 288] Therefore, it was clear the next candidates for investigation were proteins that effect FH-ligand abundance and presentation during dengue.

Factors that influence host binding by FH can be as important for AP regulation as FH itself. For example, disrupting sialic acid binding domains in the FH C-terminal region ablates FH AP regulatory capabilities which can be fatal, as in the case of aHUS and disruption of the HS binding domain increases the risk of incurable, complement mediated, blindness, AMD.[171, 186, 289, 290] Dengue increased mRNA for the proheparanase activating enzyme, CTSL relative to dengue-negative febrile disease and patient matched convalescent samples in all three studies. Increased CTSL mRNA abundance persisted for at least 7 days after symptom onset and was independent of disease severity or primary compared to secondary infection. Increased CTSL proteolytic activity, which increases heparanase activity, has been observed for WNV and DENV-NS1 treated ECs, inducing endothelial hyperpermeability.[15] However, the investigations defining HUVEC responses to NS1 only quantitated the CTSL protein and not CTSL mRNA. In another study investigating DENV infection in HepG2, CTSL mRNA was significantly increased by 1.5-fold 24 hpi and was 2-fold higher than uninfected controls by 48 hours.[291] Thus elevated CTSL mRNA in HepG2 aligns with the findings herein using cells from whole blood, although not at the same magnitude.

Excitingly, the link between CTSL and FH is not limited to heparanase activity or replicating virus. In response to cell death, Liszewski et al., (2013)[293] observed that FH is actively internalised by apoptotic cells to regulate intracellular complement activity by binding intracellular CTSL, ultimately promoting non-inflammatory phagocytosis. Intracellular complement activity is a novel topic of complement research first investigated less than a decade ago.[292, 294] These interactions could be investigated during dengue *in vitro*. First, by determining whether DENV-infection upregulates CTSL mRNA as observed in this chapter. FH and CTSL interactions could then be investigated by immunofluorescent labelling of FH and CTSL and observed by fluorescence microscopy.

As DENV infection was shown to change the relative abundance of FH different to FHR mRNA, despite a common evolutionary origin, TFs associated with immune responses were of particular interest. Therefore, promoter analysis for *CFH and CFHR1-5* were performed in an attempt to identify differences in TF binding element motifs, with the aim of elucidating why mRNA abundance for these genes was different

during dengue and severe dengue. As expected, although not reported previously by other *in silico* analyses of *CFH*, all *CFH* family genes (*CFH*, *CFHR1-5*) had multiple liver-associated TF binding sites i.e. the hepatic nuclear factor family, not previously reported by Cabezas, (2018)[43] or Ormsby (2004)[177]. Additionally, the parent *CFH* gene had the highest fold-change in mRNA of any FH protein family member, likely due to the unique presence of NF-κB binding sites (RelA) predicted by PROMO. This result is consistent with the findings of Cabezas, (2018)[43], Ormsby (2004)[177] by MATINSPECTOR and Pastor *et al.,* (2013) by electromobility shift assay.[176, 182] The activity of the NF-κB binding motif during dengue is correlated with disease severity.[157] NF-κB-mediated FH induction likely occurs downstream of multiple PRRs such as TLR-3, RIG-I and MDA5, activated by DENV infection as detailed in **Figure I-1.9**.[182] To test whether the predicted NF-κB site is responsible for FH induction during dengue, a promoter reporter construct could be used. This could be achieved using existing *CFH* promoter-reporter construct (GenBank #Y08933) containing the wild-type promoter or mutated TF domains of interest upstream of the firefly luciferase reporter gene. This construct could then be transiently transfected into cells, followed by DENV infection to detect differences in transcriptional regulation *in vitro*. To accompany this experiment, promoter-reporter constructs for *CFHR1-5* with an NF-κB site insertion could be used for comparison.

IRF-1 binding sites upstream of *CFH* were also predicted by PROMO and supported by previous analysis by MATINSPECTOR.[43, 177] The predicted IRF-1 but not IRF-2 sites in the *CFH* promoter were particularly interesting, as IRF-2 is described to compete with IRF-1, without enhancing transcriptional activity of the open reading frame.[295] Additionally, IRF-1 is known to have increased mRNA and activation in response to DENV infection which is induced by detection of the DENV genome in the endosome and cytosol.[296, 297] To determine the importance of the IRF-1 site in *CFH* during DENV-infection, a similar experiment as described for NF-κB could be performed.

The *CFHR* gene family result from duplications of the *CFH* parent gene and as such, interact with the AP utilising the domains retained from *CFH*. However, our understanding of the FHR family is still limited. In some instances, FHRs have been reported to direct FH behaviour or in-part compensate FH activity on specific tissue

surfaces and in other instances, activate the AP.[198] FHR-viral interaction specifically is an uninvestigated field of research and, considering the correlation between FH and dengue severity, relative FHR mRNA abundance in dengue patients was investigated. FH and the FHR proteins are primarily produced by the liver.[145, 161, 162] However, local concentrations of FH and FB are influenced by EC and macrophage production[298, 299] and this may therefore be true for FHRs. Thus, abundance of transcripts for FHR-1-5 should be investigated in DENV-infected MDM and EC to extend our understanding of extrahepatic FHR induction in dengue and viral infection more broadly.

FHR-1 mRNA was acutely increased during non-dengue febrile diseases but was observed as either unchanged or decreased during dengue. FHR-1 mRNA was slightly decreased in severe dengue but was independent of whether infection was primary or secondary. Induction of FHR-1 mRNA for other febrile illnesses but not dengue might contribute to AP dysregulation in dengue and is an interesting target for future investigation. Therefore, mRNA abundance for FHR-1 clearly opposes the pattern of FH mRNA and the TF binding elements predicted upstream of each gene were assessed in an attempt to delineate the role and importance of these sites. A notably different TF binding site for *CFHR1* was POU2F2, only predicted for *CFHR1* and *CFHR5*. POU2F2 binding regions are typically found in immunoglobulin promoters and is highest in B-cells, suggesting its activation is linked to lymphocyte activity.[300] Interestingly, B-cells but not T-cells or monocytes produce FHR-1, but this production decreases following B-cell activation.[301] POU2F2 enhances transcriptional activity of the promoter regions it binds, therefore, if it is activated would likely cause FHR-1 mRNA induction. The *CFHR1* promoter was unique from other promoters by lack of many TF binding motifs found in most of the *CFHR* genes (c-Myb, ER-α, Pax-5, and PXR-1:RXR-α). The transcription factors c-Myb and Pax-5 are both transcription factors essential for B-cell differentiation, maturation, and proliferation. Aside from supporting an adaptive immune response to infection, the role of these transcription factors during dengue is unclear. RXR-α is an inhibitory transcription factor that prevents access to the promoter region of genes it binds. Activated PXR-1 can enhance promoter activity of RXR-α sites and is activated in response to dexamethasone, xenobiotics, and steroids, where dexamethasone has been demonstrated to induce FH mRNA.[302, 303] As PXR-1:RXR-α activation typically occurs

in response to sex hormones and xenobiotics, it is unlikely DENV-infection promotes FH mRNA through this pathway. FHR-1 mediated AP regulation and activation appear context specific. *In vitro*, FHR-1 is described to prevent C5 convertase activity and MAC formation.[254, 304, 305] On the other hand, FHR-1 will compete with FH for DNA of apoptotic cells and for pathogens attempting to bind FH and evade innate immune responses (**Figure I-11)**.[197, 205, 257, 306] Therefore, it is difficult to determine whether raising FHR-1 abundance, as occurs in other febrile diseases would aid AP regulation, or worsen disease in the context of dengue.

The influence of FHR-1 on dengue severity could be tested in dengue patients without the need for medical intervention. Discussed in further detail in **Chapter VI**, approximately 10% of the global population are carriers of a deletion for *CFHR1* and *CFHR3* (*CHFR3-1Δ)* which occurs less commonly in East Asia and is virtually not present in Japanese and South American populations.[203] However, homozygous *CFHR3-1Δ* occurs in about 1 in 3 people with Sub-Saharan genetic ancestry and is a region of the world impacted by dengue.[2, 203] Multiplex ligand array kits already exist to screen for *CFHR3-1Δ* and could be used to determine the copy number of *CFHR1* (and *CFHR3*).

With a sufficiently large cohort and GWAS and dengue patient disease scoring could be paired, and statistical analyses performed to determine whether the presence of FHR-1 modulates the risk of developing severe dengue. Existing GWAS performed by Beatriz *et al.,* (2014)[307], Xavier-Carvalho *et al.,* (2017)[308], Whitehorn *et al.,* (2013)[309] or Pare *et al.,* (2020)[310] for example would not assist in answering these questions, as copy number variation of *CFHR3-1Δ* requires targeted analysis by multiplex ligand array described in detail in **Section VI.8**. Correlation between *CFHR3-1Δ* and dengue severity has been assessed once, once as in Kraivong *et al.,* (2013)[243]. However, this study failed to recognise the importance of ethnicity in investigating *CFHR3-1Δ,* as it was performed in a Thai population. Importantly, homozygous *CFHR3-1Δ* rarely occurs in people of Asian descent and accordingly, homozygous *CFHR3-1Δ* was only observed for 0.8% of participants, suggesting this study lacked the power to assess this relationship.[203] Interestingly however, the studies performed by Beatriz *et al.,* (2014) and Xavier-Carvalho *et al.,* (2017) both determined African ancestry in Cuban, Brazilian and Columbian populations was protective of severe dengue. This supports

the possibility that FHR-1 influences dengue severity as Holmes *et al.,* (2013) observed people with African ancestry had the highest prevalence of *CFHR3-1Δ*.

Abundance of FHR-2 mRNA was observed as reduced during the acute phase of dengue but independent of dengue severity. FHR-2 mRNA was reduced further in primary dengue patients relative to secondary dengue. FHR-2 downregulation was not unique to dengue, as it also occurred in DENV PCR-negative febrile patients. Analysis of the *CFHR2* promoter predicted a STAT1β binding site which was only found in *CFHR2* and *CFHR5* and may have been responsible for decreased mRNA, as it was not found upstream of *CFH*. STAT1β is a splice variant of STAT1 and binds but cannot increase transcription of a gene due to lack of a transactivation domain, potentially blocking promoter site binding for other TFs.[311] A promoter reporter system described above but using the *CFHR2* promoter could be used to test this hypothesis. To directly test whether STAT1β binding inhibits *CFHR2* promoter activity, with the aid of this construct, site directed mutagenesis of the TF binding site or addition of the selective STAT1/STAT1β inhibitor, fludarabine[312] could be used. FHR-2 AP-interaction is not yet well defined, but it does not compete with FH, and has been observed to reduce AP activation by interfering with C3-convertase activity.[197] Therefore, decreased FHR-2 mRNA in response to infection and febrile disease may prevent C3-convertase activity from being undermined by circulating FHR-2. Potentially, elevating FHR-2 may support AP regulation during dengue by targeting the positive feedback system C3-convertases amplify.

No change in relative FHR-3 mRNA abundance was detected when comparing primary and secondary dengue patients or dengue severities and changes in FHR-3 mRNA abundance were inconsistent across GSE28405, GSE40165 and GSE28988. As the data are in total conflict, no conclusion for FHR-3 during acute phase dengue compared to homeostatic (convalescent) mRNA abundance can be drawn without further investigation. GSE28405 and GSE28988 were performed by the same research team two years apart, in Singapore, using the same sample collection methods and definitions for acute and convalescent disease.[92, 272] Thus, there are no clear disparities in experimental design that would account for opposing results.

The role of the elements predicted upstream of the *CFHR3* gene are difficult to interpret in the context of conflicting results between the three comparable GEO datasets and no promoter binding element presence or absence was unique to the *CFHR3* promoter. Therefore, the role FHR-3 performs during dengue remains to be determined and requires further investigation.

Relative FHR-4 mRNA abundance was not increased during the acute phase of dengue or dengue-negative febrile disease compared to homeostatic concentrations. Additionally, FHR-4 mRNA was independent of whether the patient was experiencing primary or secondary dengue infections but was increased during severe disease. There was no unique element of the *CFHR4* promoter region with respect to immune-associated TFs. *CFH*, *CFHR4* and *CFHR5* were all predicted to have c-Myb promoter binding sites, and transcripts from each of these genes increased during severe dengue. The c-Myb sites was predicted to occur at least once per 1200 bp by chance alone due to the short length of the consensus sequence (5'-YAAC[GT]G-3') and could be a false discovery. Although, c-Myb sites were not predicted in transcripts that were unchanged or decreased during severe dengue (FHR1-3). Additionally, c-Myb is a protooncogene confined to activity in stem cells, except in the cases where it causes cancer and so, is probably not the cause of gene induction during dengue.[313] FHR-4 can provide a scaffold for C3b deposition, promoting C3-convertase formation, amplifying complement AP activation feedback mechanisms.[211] Therefore, unchanged and decreased FHR-4 mRNA likely benefits patients during dengue and conversely, increased FHR-4 mRNA in severe dengue patients is likely deleterious as AP activity correlates with dengue severity.[201, 314] The increase in FHR-4 mRNA unique to severe dengue patients may be interesting to investigate as a prognostic biomarker, which is still needed for dengue.[13]

FHR-5 mRNA abundance did not increase during acute phase dengue (GSE28405 and GSE40165), although it did increase during acute dengue-negative febrile illness. In GSE28988, FHR-5 mRNA was below the limit of detection. FHR-5 mRNA was elevated in patients who developed severe disease. FHR-5 deposits are associated with complementopathy that typically targets the kidney.[206, 315] Kidney involvement during severe dengue has been documented, occurring in more than 10% of cases[316] and might be affected by patient FHR-5 production. Additionally, at physiological

concentrations, *in vitro*, FHR-5 can assist C3b deposition and so, is predominately associated with complement AP activation.[256] As FHR-5 mRNA was below the limit of detection in one study, which was not observed for any other gene investigated in this chapter, FHR-5 mRNA abundance in whole blood cells may border the lower limit of detection. This could be easily tested through quantitation of FHR-5 mRNA by RT-qPCR for cells found in whole blood. As with FHR-4, the increase in FHR-5 mRNA unique to severe dengue patients has exciting prospects for consideration as a biomarker of severe dengue in the future.

The *CFHR5* gene was predicted to contain every immune-associated TF binding site detected in *CFH* or *CFHR1-4* with the exception of the NF-κB subunit, RelA. As FHR-5 mRNA did not apparently increase during DENV, potentially inhibitory actions of TFs identified such as IRF-2 and STAT1β may compensate the enhancer activity of other TFs identified such as IRF-1. FHR-5 is important for opsonising cellular debris and the surface of apoptotic and necrotic cells and so, it was surprising no apoptosis-associated TF binding sites were predicted for *CFHR5* such as ATF-2/3 or SP1 which were observed in *CFH*, *CFHR1* and *CFHR4*.

The interactions between FH binding and the HS and sialic acid-interacting proteins investigated in this chapter are depicted in **Figure III-9**. *NEU1-4* encode for neuraminidases that localise to different cellular compartments and have different cleavage substrates **(Figure III-9)**.[317-321] DENV-NS1 is known to acutely induce neuraminidase activity for NEU1-3, disrupting the extracellular endothelial glycocalyx in ECs *in vitro*.[15] Lysosomal neuraminidase NEU1 mRNA was 2 to 3-fold higher in dengue patients in all three dengue cohorts (GSE28405, GSE40165, GSE28988) comparing patient matched acute and convalescent samples but independent of whether patients presented with primary or secondary dengue. During dengue, abundance of mRNA for NEU2 and NEU3 were unchanged and inconsistent, respectively. From these results, it is difficult to determine the relevance of the role these proteins perform during dengue. NEU1 does not cleave cell membrane sialic acid but increased mRNA was observed herein and has been reported *in vitro* in DENV-NS1 treated cells.[15] The effect of NEU1 lysosomal sialidase activity or increased NEU1 mRNA abundance on sialic acid during DENV infection could be tested by reducing nascent NEU1 mRNA through siRNAs or decreasing NEU1 activity

with oseltamivir. Cells could then be infected with DENV and differences in sialic acid quantitated by IF or the novel sialic acid quantitation assay developed in **Chapter IV**. NEU4 mRNA was decreased in dengue-negative febrile controls relative to dengue patients but was not lower than abundance during convalescence. The marginal decrease in NEU4 does not have a clear implication for physiological changes but does demonstrate a difference in expression of NEU1-3 from NEU4. Additionally, this demonstrated that NEU4 mRNA abundance is influenced by primary compared to secondary dengue infection. Thus, although NS1 acutely induces NEU1-3 proteins *in vitro* there is no evidence in whole blood of dengue patients that NEU2-4 mRNA transcripts are substantially different compared to homeostatic levels. Therefore, changes in the transcript abundance of NEU2-4 are unlikely to make a significant impact on the behaviour of FH-sialic acid binding but may instead be effected by NEU2-4 activity. HS is anchored to proteoglycans on the extracellular membrane which are degraded by enzymatic processes.[322, 323] During dengue, vascular insufficiency is worsened by degradation of the glycocalyx, which is in-part made up by HS and other GAGs, which are also the ligands for FH host-cell recognition.[324] HSPG2 is an extracellular HS proteoglycan that forms part of the glycocalyx, as a process of glycocalyx maintenance, lysosomal HS degradation is performed by HGSNAT **(Figure III-9)**.[325] No consistent change for HGSNAT was observed between the studies and it is therefore difficult to determine the physiological relevance of HGSNAT abundance during dengue. HSPG2 mRNA was increased during acute disease in two of three studies, was higher than dengue negative febrile controls and independent of disease severity or primary compared to secondary infection. The data suggest HSPG2 mRNA would usually be decreased during other febrile illnesses, but not dengue. DENV uses HS proteoglycans as an attachment factor.[326, 327] This may therefore explain why other febrile diseases cause downregulation of HSPG2, but DENV does not. As HS proteoglycan binding is a trait of other flaviviruses, it may prove valuable to investigate changes in transcription for HSPG2 in response to other viruses such as ZIKV, JEV and WNV to determine whether these also act in opposition to non-flavivirus induced febrile disease.

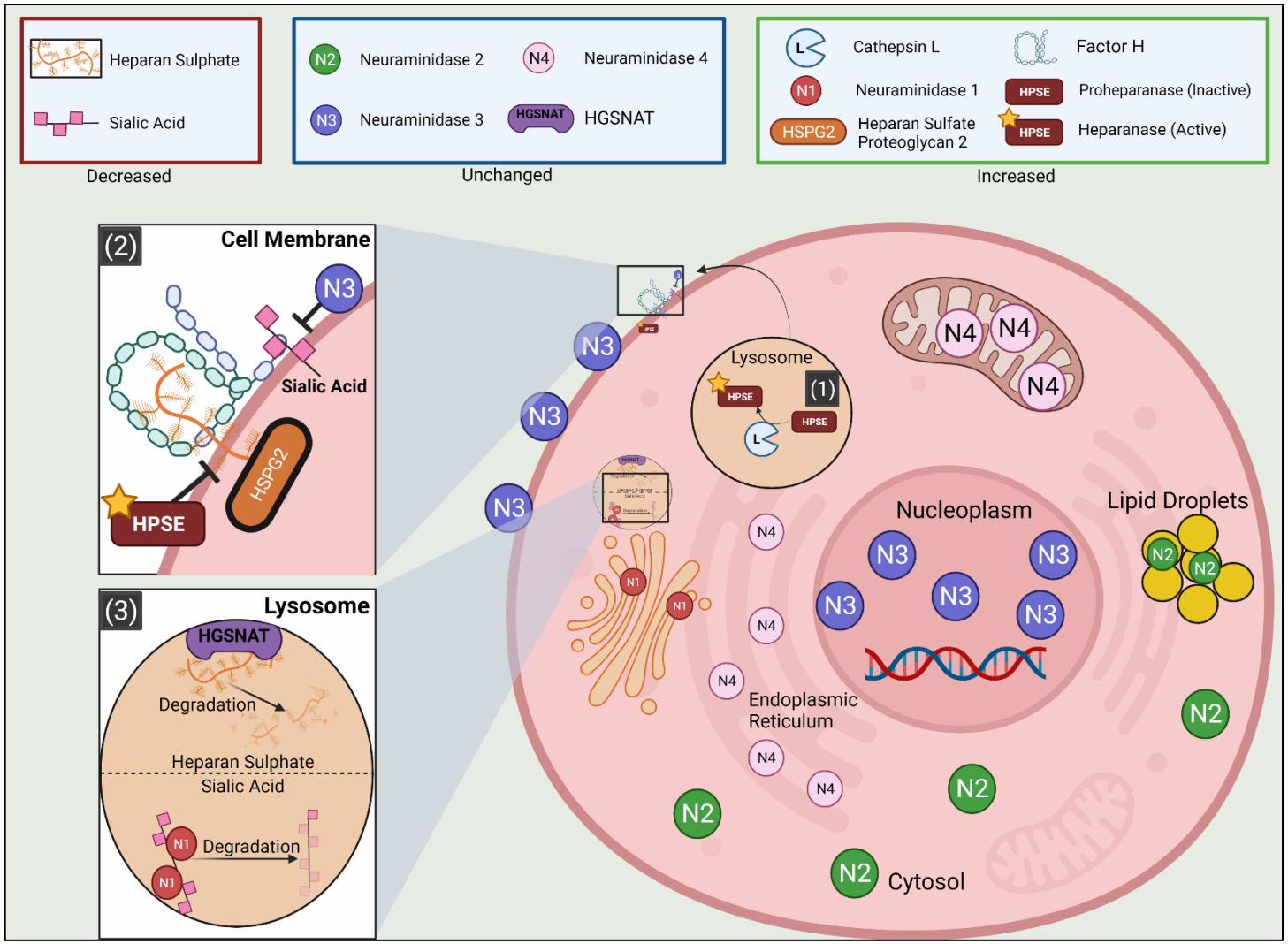

Figure III-9. The location and predicted change in HS and sialic acid-interacting proteins based on the results of GEO dataset mining.

Based on the results of this chapter, HS and sialic acid-interacting proteins are hypothesised to change (decreased in the red box, unchanged in the blue box and increased the in green box). Neuraminidases (sialidases) exist in 4 forms that differ by location in the cell. Neuraminidase 1 is primarily found within the lysosome, neuraminidase 2 is found within the cytosol and in lipid droplets, neuraminidase 3 is found in the nucleoplasm and also acts extracellularly, neuraminidase 4 is found within the mitochondria. **(1)** Heparanase is expressed in an inactive state (proheparanase) and is activated by cytosolic cathepsin L. **(2)** Following activation, heparanase is secreted and can act in the extracellular space. Factor H binds the extracellular membrane by HS and sialic acid but heparanase and neuraminidase 3 will cleave HS and sialic acid from the membrane respectively, preventing FH binding. **(3)** Turnover of HS and sialic acid is mediated within the lysosome by HGSNAT and neuraminidase 1, respectively. HGSNAT = Heparan-α-glucosaminide N-acetyltransferase.

HPSE mRNA is translated into proheparanase, which requires proteolytic activation by CTSL to function **(Figure III-9)**.[260] Lower HPSE mRNA than febrile controls and an increase for only one of three dengue cohorts was surprising, as HPSE mRNA increases in ECs treated with TNF-α and IL1β, both of which are increased during dengue.[328] Additionally, DENV-NS1 acutely increases HPSE activity *in vitro* but, as with neuraminidases, it is possible that dengue-induced HPSE activity is mediated post-translationally, not by the transcription of proheparanase mRNA. CTSL activates heparanase and the observed increase in CTSL mRNA would still increase the amount of active heparanase, even without increased nascent proheparanase. It is important to note that the changes in mRNA abundance for sialic acid and HS interacting genes were confined to whole blood cells and does not capture EC response where glycocalyx breakdown occurs. Additionally, DENV-infected cells compose only a small percentage of circulating cells captured by whole blood collection[282] and therefore, small changes may be difficult to detect without first removing cells that are not permissive to DENV.

The observation of changes in mRNA abundance, despite typically low rates of infected cells in whole blood is suggestive of transcriptional changes made by cells in response to the stimuli produced during infection. This is in contrast to a response to intracellular virus and the difference between these stimuli was examined further in **Chapter V**. Future analyses in ECs should investigate the glycocalyx and vascular permeability or hepatic cells *in vitro* for investigating systemic complement production. In combination with the results of this chapter, these analyses would form a more wholistic understanding of the complementome and FH interactome during dengue.

Mining publicly available data has inherent limitations. For example, altered conditions across datasets complicates comparisons across experiments. Furthermore, study parameters such as age range, gender proportions and incomplete patient assessment may confound outcomes for specific genes of interest. Additionally, it is unclear whether the inclusion of younger dengue patients in two of four cohorts would have affected mRNA abundance but may also provide explanation for the differences observed.

Other methods for comparing existing microarray data include GenomeStudio and Beeline.[329, 330] These tools would equally be useful to mine publicly available data, however use of GEO2R has advantages. Namely, GEO2R analysis has a robust interface tested by thousands of users, ease of use and direct access to NCBI datasets.[263]

Correlates of mRNA abundance are summarised in **Figure III-10**. To summarise the correlates of mRNA abundance with dengue and severe dengue for the genes investigated in this chapter: Healthy individuals have higher FHR-2 mRNA than dengue and severe dengue patients. Dengue patients had elevated mRNA for RSAD2, FH, FB, CTSL, HSPG2 and NEU1. Severe dengue patients also have higher FH and FB as well as a unique increase in FHR-4 and FHR-5 mRNA. Changes in HPSE, HGSNAT, NEU2-4, FHR-1 and FHR-3 were either not consistently observed or conflicted between studies.

III.4 Summary

The available dengue patient data mined by GEO2R were used to assess induction of mRNA for AP members (FH and FB), the FHR family (FHR-1-5) and FH-ligand effecting genes (HGSNAT, HPSE, HSPG2, CTSL and NEU1-4). These data provided a foundation for hypotheses to test in later chapters *in vitro*. In dengue, viperin, FH, FB, HSPG2, CTSL and NEU1 mRNA abundance increased in whole blood cells, likely by inducing transcription, although increasing mRNA stability or dampening post-transcriptional regulation are another possibility. Increased FH and FB mRNA following DENV infection suggests that some post-transcriptional process or processes are influencing the fate of nascent FH proteins but not FB. Such changes might include cell-stress induced protein degradation, redirection by CTSL-mediated mechanisms or maybe prevention of FH mRNA translation entirely. In future chapters, the dogma of expression from transcription to translation and post-translational ligand binding was followed, quantitating the abundance of FH and ligand-bound FH in an attempt to characterise FH expression and localisation during DENV infection.

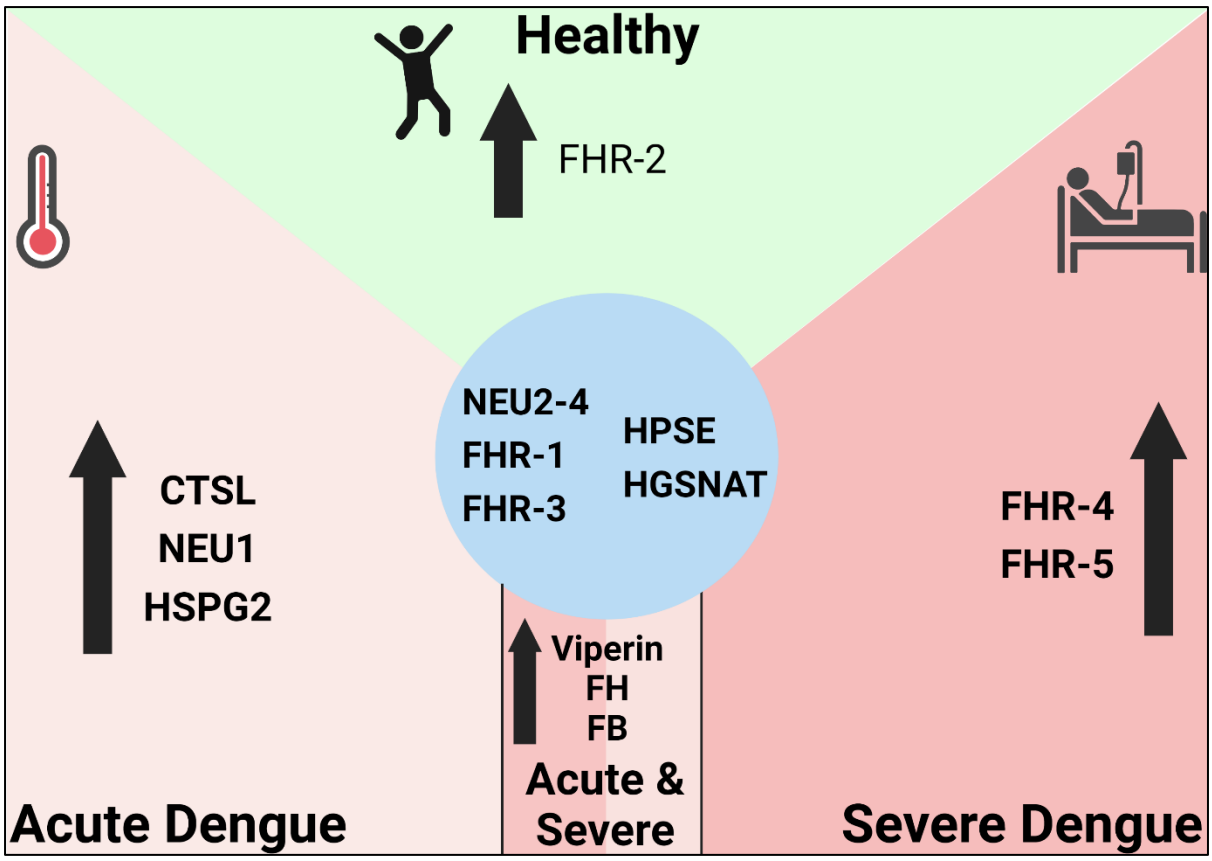

Figure III-10. Dengue upregulates FH, FB and viperin and severe dengue upregulates FHR-4 and FHR-5.

The observed changes in relative mRNA abundance of complement and GAG-associated genes in healthy, acute dengue (also referred to as dengue) and severe dengue patients as determined by datamining existing GEO datasets. Changes in mRNA abundance associated with disease state were observed in at least two of three studies. mRNA for genes with no significant difference, conflicting results between studies or changed in only one data set are listed in the blue circle as inconclusive. FHR-2 mRNA is higher in healthy individuals than dengue patients, CTSL, HSPG2 and NEU1 mRNA increase during dengue independent of disease severity. RSAD2 (viperin) FH and FB have increased mRNA during dengue which increases further with disease severity. FHR-4 and FHR-5 mRNA do not increase during dengue but are increased in severe dengue patients

CHAPTER IV

DEFINING FH PRODUCTION AND LOCALISATION IN DENV INFECTED CELLS

IV.1 Introduction

The observations in **Chapter III** demonstrated FH mRNA induction occurs in whole blood cells of dengue patients. However, increased FH mRNA would be expected to cause increased circulating FH protein, which does not occur. Instead, circulating FH is unchanged or reduced in dengue and severe dengue patients respectively.[217] This phenomenon of unchanged FH protein, in spite of increased FH mRNA in patients is mirrored by both *in vitro* and *in vivo* models of DENV infection.[44] In DENV-infected MDM and EC, FH protein in the supernatant is unchanged despite induced FH mRNA. In DENV-infected mice, FH is unchanged in circulation, despite induced hepatic FH mRNA.[176] Further definition of FH production and localisation *in vitro* is hampered by the availability and practicality of primary cell line use, therefore a more robust immortalised cell line that reproduces the responses of MDM and EC to DENV was sought and HeLa selected as a model. Using HeLa, FH mRNA length and protein size were defined further to assess the possibility of abortive transcription, proteasomal degradation or FH isoforms interfering with previously employed methods of FH mRNA and protein quantitation. These methods were used to test the hypothesis that DENV infection induces full length FH mRNA and protein. The hypothesis that cell membrane augmentation retained FH to the cell membrane was tested also.

Additionally, prior data has shown increased and cell-associated FH in DENV-infected MDM, by flow cytometry.[44] Here, the FH-binding substrates, sialic acid and HS, are both acutely decreased in response to DENV-NS1 on the endothelial glycocalyx.[15] These ligands were though tot also be affected by the increased CTSL and NEU1 reported in **Chapter III** and thus, were evaluated. The distribution of the FH protein in and on DENV-infected and uninfected bystander cells was also defined using high resolution IF and confocal microscopy.

IV.2 Results

IV.2.1 DENV-infection in HeLa cells induces FH mRNA and protein reflective of MDM and EC

Analysis of the interaction between DENV and FH in MDM as previously undertaken in our laboratory is expensive, suffers inter-donor variability and is difficult in obtaining large cell numbers for some experiments.[44] Thus a cell-line that reflects the same responses as previously defined in primary macrophages was sought. Four cell lines were infected with DENV and had RNA harvested 48 hpi. RT-qPCR was performed to quantitate mRNA for the housekeeping gene cyclophilin, FH and DENV RNA. DENV replication was confirmed in HeLa, Huh7.5 and U937, as determined by qPCR. However, HEK293 infection demonstrated low normalised DENV RNA **(Figure A.1)**. As DENV RNA was readily detectable, and these cells did not produce detectable levels of FH, HEK293 susceptibility to DENV-infection was not investigated further.

The monocytic cell line U937 and HEK293 cells did not produce detectable amounts of FH mRNA or protein in uninfected or DENV-infected cells were not considered further **(Figure IV-1A-B)**. DENV-infection of HeLa induced FH mRNA, but secreted FH was unchanged compared to uninfected controls, as previously observed in primary MDM **(Figure IV-1A-B)**. The liver, is the main source of circulating FH *in vivo*, and although the liver is not a primary site for DENV-infection and evidence for replication of DENV in the liver is conflicting, the effect of DENV on FH production from Huh7.5 cells was defined.[331, 332] In our hands, Huh7.5 produced approximately 3-fold higher basal levels of secreted FH relative to HeLa (6.9±0.9 vs 2±1.2 μg/mL respectively). Both uninfected Huh7.5 and HeLa produced secreted FH at much higher concentrations than previously reported concentrations for primary MDM and HUVEC (600 ng/mL and 60 ng/mL respectively). In contrast to HeLa cells, a significant decrease in FH mRNA was observed that was accompanied by a significant reduction in FH protein in the supernatant of DENV-infected Huh7.5 cells **(Figure IV-1A-B)**. Consistent with this, IF staining for FH in DENV-infected HeLa and Huh7.5 showed an increase and reduction, respectively in signal for FH. Additionally, IF staining confirmed the presence of intracellular dsRNA as an indication of infection for both HeLa (approximately 40% dsRNA positive) and Huh7.5 (~100% dsRNA positive cells). **(Figure IV-1C)**.

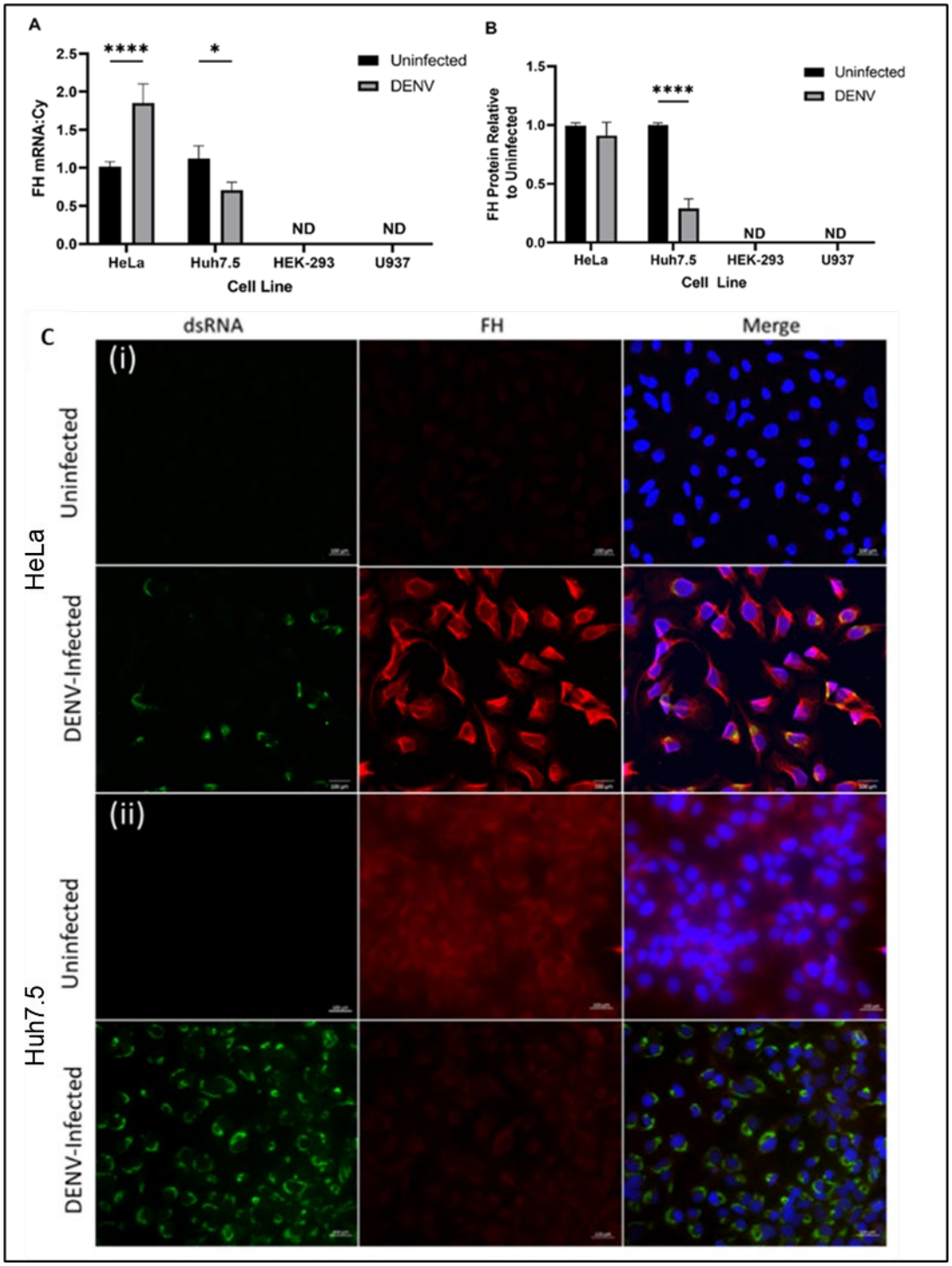

Figure IV-1. FH mRNA and cell-associated FH increase in HeLa but decrease in Huh7.5 following DENV infection.

(A, B, C(i)) HeLa and **(A, B and C(ii))** Huh7.5 were uninfected or DENV-infected (MOI=1) and incubated for 48 hr. Total cellular RNA, or total protein lysates and supernatant were collected and analysed for **(A)** FH mRNA by RT-qPCR normalised to cyclophilin and **(B)** supernatant FH by ELISA relative to uninfected controls of the respective cell line. Results of qPCR and ELISA were normalised to uninfected cells of a cell type and represent the mean ± standard error for pooled triplicate samples from three independent infections. *=p<0.05, ** = p<0.005, ***=p<5x10^{-3}. Student's *t*-test. **(C)** Cells were fixed at 48 hr and stained for nuclei (blue), double stranded RNA (green) and FH (red). Images were captured with an Olympus AX70 Fluorescence microscope at 40x magnification and are representative of three independent experiments. DENV = Dengue Virus, FH = Factor H.

IV.2.2 Full length FH protein is detectable in DENV infected cells

The results of **Figure IV-1C** suggests cell-associated FH increases in HeLa following DENV-infection, consistent with the unchanged levels of FH quantitated in the cultured supernatant. Thus, FH was quantitated within cell lysates by ELISA and demonstrated an increase in cell-associated FH protein, consistent with **Figure IV-1C**. **(Figure IV-2A)**. Uninfected cells contained 1.2 ± 0.15 µg/mL FH within the lysates. The presence of full-length (approximately 155 kDa) FH protein in the supernatant and cell lysates from uninfected and DENV-infected cells was observed **(Figure IV-2B).** In contrast to ELISA, levels of full-length FH appeared comparable or slightly higher in uninfected cell lysates relative to those from DENV-infected cells by Western blot **(Figure IV-2A-B)**. However, equivalent amounts of purified FH to that previously quantitated in uninfected and DENV-infected samples by ELISA (10 and 25 ng per well respectively), could not be discerned by Western blot. This demonstrates that Western blot is not sensitive enough to quantitate a 2.5-fold difference, as seen by ELISA **(Figure A.2)**. Additionally, FH-immunoreactive species at approximately 50-55 kDa were observed in cell lysates but not in human serum **(Figure IV-2B)**. The appearance of these bands was spurious, occurring in 3 out of 5 Western blots even for analysis of the same sample **(Figure A.2)**. Based on this, the 50-55 kDa bands were considered non-specific cross-reactive proteins and not pursued further.

Potentially, intracellular FH may not have been released if it was not functional, perhaps due to targeting to the proteasome. Therefore, the effect of inhibiting the proteasome was investigated. Widely utilised proteasome inhibitors MG132 and bortezomib, were used to determine if this treatment would increase FH protein released into the cell culture supernatant. FH mRNA was still induced by DENV infection in the presence of bortezomib and MG132 **(Figure IV-2C-D)**, although MG132 had significantly decreased secreted FH protein in both uninfected and DENV-infected cells relative to vehicle controls (0.1% v/v DMSO). Neither MG132 nor bortezomib treatment significantly affected secreted FH protein in comparison to drug-treated, uninfected cells **(Figure IV-2C-D)**.

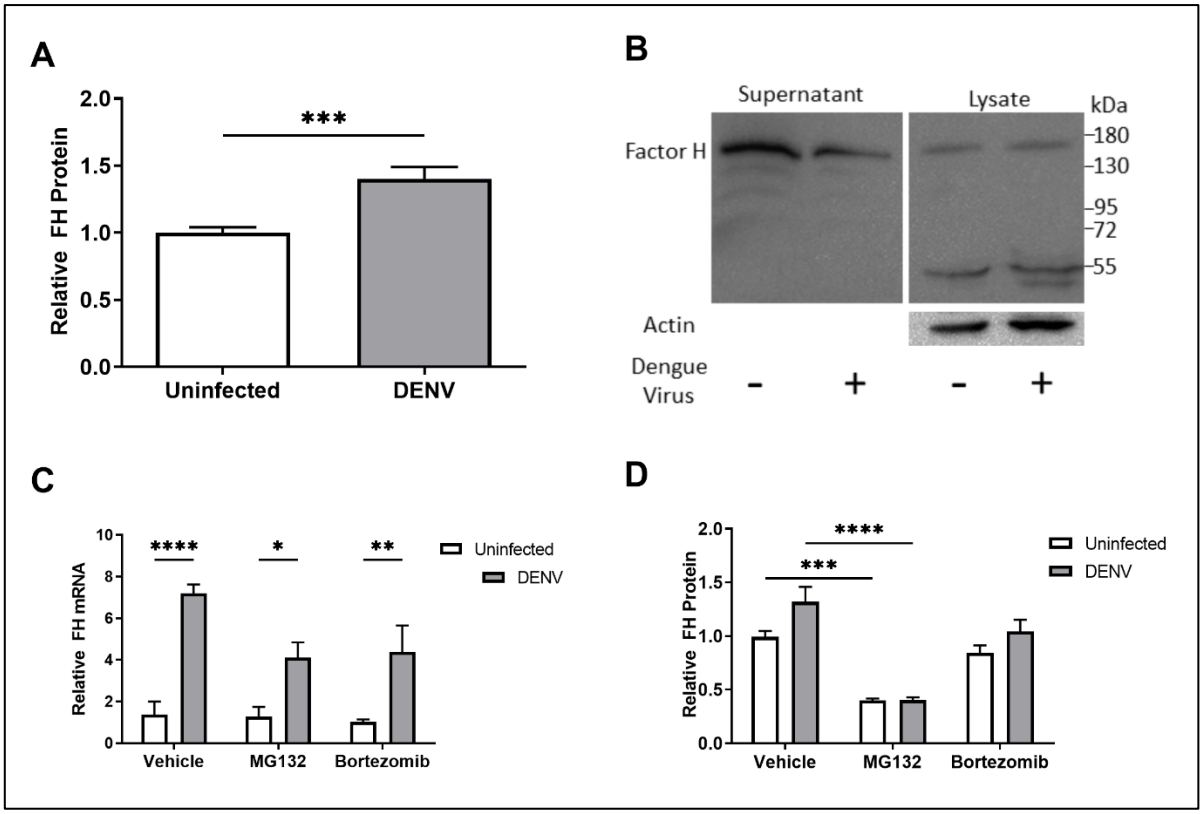

Figure IV-2 Intracellular FH protein is increased but the proteasome does not impair FH secretion.

HeLa were uninfected or DENV-infected (MOI=1) and incubated for 48 hr. Supernatant and cell lysates were collected and analysed for **(A)** FH within lysates by ELISA normalised total protein, expressed relative to uninfected controls and **(B)** FH and actin by SDS-PAGE and Western blot, using 30 µl of supernatant or 30 µg of intracellular total protein. Molecular weight markers are indicated, and bound complexes were visualised by chemiluminescence. **(C, D)** HeLa were uninfected or DENV-infected (MOI=1) for 24 hr and then treated with the vehicle (DMSO (0.1 % v/v)), MG132 (5 µM) or bortezomib (5 nM) and incubated for a further 24 hr. Total cellular RNA and supernatant were collected. **(C)** FH mRNA for uninfected and DENV-infected cells was quantitated by RT-qPCR and values were normalised to cyclophilin and expressed relative to uninfected vehicle controls; **(D)** FH in uninfected and DENV-infected cell supernatants was quantitated by ELISA and are expressed relative to vehicle controls. Results represent the mean ± standard error for (n=4) samples, representative of three independent infections. * = $p < 0.05$, ** = $p < 0.005$, *** = $p < 5x10^{-3}$ **** = $p < 5x10^{-4}$. One-way ANOVA with Bonferroni corrections.

IV.2.3 Full length FH mRNA is induced by DENV infection

The FH mRNA molecule is long (4 kb) and can be alternatively spliced to produce a smaller 42 kDa protein, FH-Like-1 (FHL-1) (1.4 kb mRNA) that is identical to FH mRNA between SCR1 and SCR7 but contains a unique 4 amino acid terminus **(Figure IV-3A)**. Additionally, there is high homology between FH_{SCR20} and FHR-1_{SCR5} **(Figure IV-3A)**. Results in **Section IV.2.1** quantitated FH mRNA by detection of FH_{SCR2}, which was previously used for MDM and would also detect FHL-1 transcripts and potentially short, mRNA degradation products **(Figure IV-3A)**. To determine if DENV-infected cells produce full-length FH mRNA, or mRNA for FHL-1 and FHR-1, primers were designed to target the FHL-1 3' UTR and unique tail, the 5' terminus found in both FH_{SCR2} and FHL-1_{SCR2}, the unique centre of FH transcripts (Exons 9/11), the 3' terminus found in both FH_{SCR20} and FHR-1_{SCR5} and the unique 3' region of FHL-1_{SCR7}. The FH_{SCR20} primers will detect both FH and FHR-1 mRNA due to high sequence homology, unlike in any other FHR and thus, Exon 9/11 primers only are unique to FH. Therefore, FHR-1 Exon 1-4 primers were designed and specifically detect FHR-1 (both FHR-1A and FHR-1B) mRNA to confirm qPCR using FH_{SCR20} was not confounded by the presence of FHR-1. **(Figure IV-3A)**. Results from each of the three FH primer sets demonstrated a significant increase in FH mRNA in DENV compared to uninfected HeLa cells **(Figure IV-3B)**. Although PCR amplification of FHL-1 and FHR-1 was successful from positive control samples **(Figure A.3)** FHL-1 and FHR-1 mRNA were not detected in uninfected or DENV-infected HeLa. Additionally, the fold-increase in FH mRNA was comparable across all three FH primer sets **(Figure IV-3B)**. Thus, increases in cDNA containing regions that bind the FH_{SCR2}, Exon 9/11 and FH_{SCR20} primers reflect increases in full-length FH mRNA and not FHL-1 or FHR-1 mRNA.

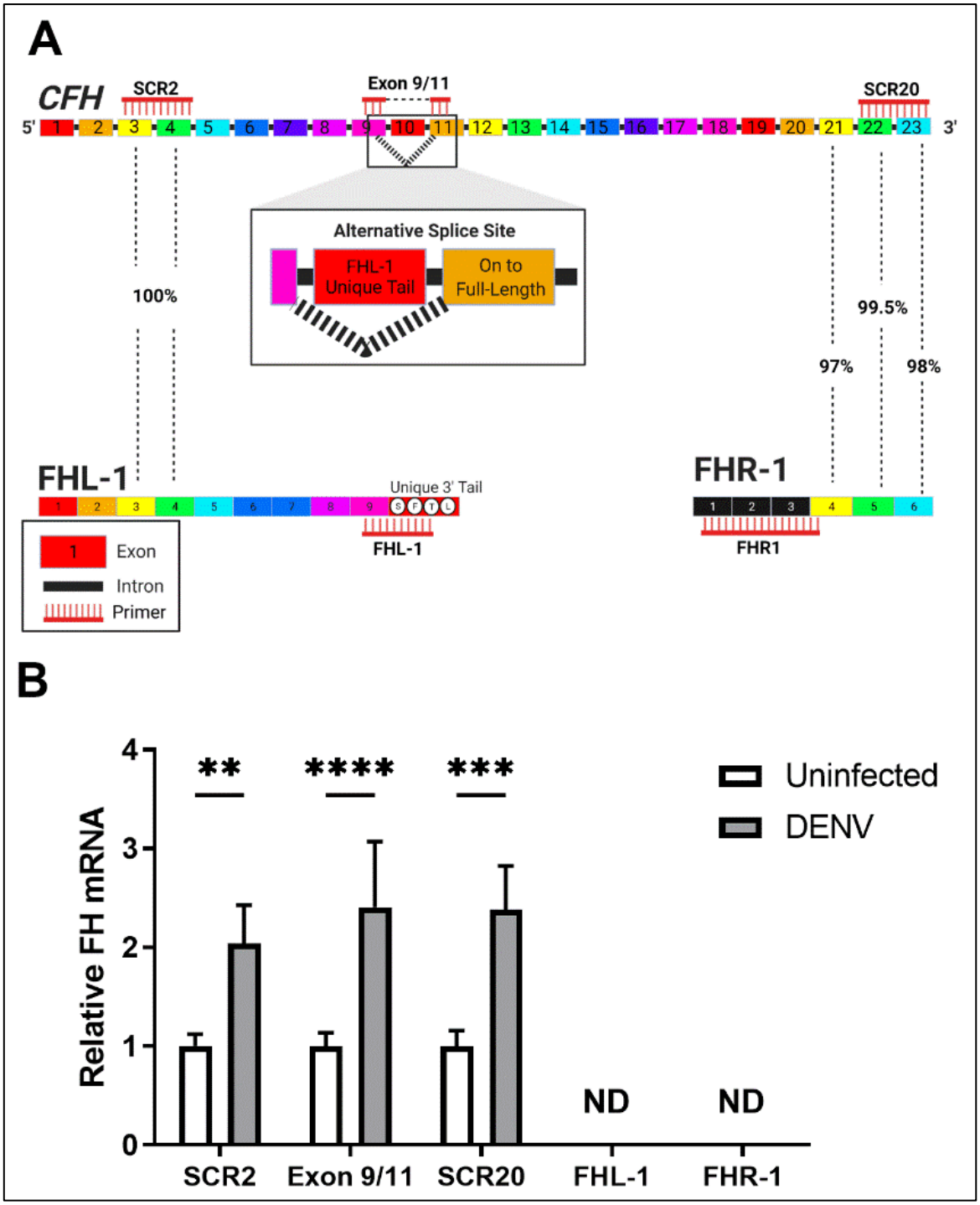

Figure IV-3. Full length FH mRNA is increased in DENV-infected cells but mRNA for FHL-1 and FHR-1 are not detectable.

(A) The *CFH* gene can produce a transcript for FH which will be detected by primers for FH_{SCR2}, Exons 9/11 and FH_{SCR20}. The FH transcript can be alternatively spliced to produce FHL-1, which will be detected by primers for FH_{SCR2} and uniquely by FHL-1 primers targeted at the 3' tail. FHR-1 (both isoforms FHR-1A and FHR-1B) mRNA is transcribed by *CFHR1* (CFHR-1A shown) and can be detected by FH_{SCR20} primers due to high sequence identity with FHR-1_{SCR5-6}, and uniquely targeted by primers for FHR-1 (both isoforms) Exons 1-4. **(B)** mRNA was quantitated in uninfected and DENV-infected HeLa by RT-qPCR using primers for FH_{SCR2}, Exons 9/11, FH_{SCR20}, FHL-1_{SCR7} and FHR-1_{SCR1-4}. Values were normalised to cyclophilin and expressed relative to uninfected FH_{SCR2} abundance. Results represent the mean ± standard error for pooled triplicate samples, from three independent infections. SCR = Short Consensus Repeat, ND = not detected, FH = factor H, FHL = Factor H like, FHR = Factor H related, DENV = Dengue virus. ** = $p<0.005$, *** = $p<5\times10^{-3}$, **** = $p<5\times10^{-4}$. One-way ANOVA with Bonferroni correction.

IV.2.4 Development of a novel assay for quantitating extracellular sialic acids

Sialic acid and HS are the primary ligands for FH on the surface of healthy cells.[143] As increased cell-associated FH was observed following DENV infection in **Section IV.2.1**, a method for quantitating the amount of sialic acid on the cell surface was sought that was cost- and time-efficient. Previous experiments investigating the amount of sialic acid detected during DENV infection used IF imaging to produce qualitative and quantitative data. Rather than microscopy, a microplate reader was used to quantitate the fluorescence produced by sialic acid binding to the FITC-conjugated ligand. Fluorescence at ex_{352}/em_{461} produced by Hoechst-33342 stained nuclei was then used to normalise sialic acid values against cell number.

Fluorescence spectral overlap was evaluated, and Hoechst-33342 fluorescence at ex_{352}/em_{461} was 90-fold higher in cells stained with Hoechst-33342 than cells without Hoechst staining. Addition of FITC-conjugated sialic acid binding lectin did not affect fluorescence at ex_{352}/em_{461} in cells concomitantly stained with Hoechst-33342. A small but significant increase in fluorescence at ex_{352}/em_{461} was observed in cells treated with sialidase and concomitantly stained with Hoechst-33342 and sialic acid-binding lectin **(Figure IV-4A)**. The sialic acid-binding lectin was confirmed to fluoresce at ex_{488}/em_{525}, independent of Hoechst-33342 staining, which was significantly decreased in neuraminidase pre-treated cells **(Figure IV-4B)**.

HeLa were cultured and then treated for one hr with sialidase from 3 mIU/mL to 90 mIU/mL and viewed by fluorescence microscopy to validate that FITC-labelled sialic acid binding lectin fluorescence was influenced by the presence of sialic acid. A distinct decrease in fluorescence at ex_{488}/em_{525} was evident as the concentration of sialidase increased, although, the microplates used were not optimal for microscopy **(Figure IV-4C)**.

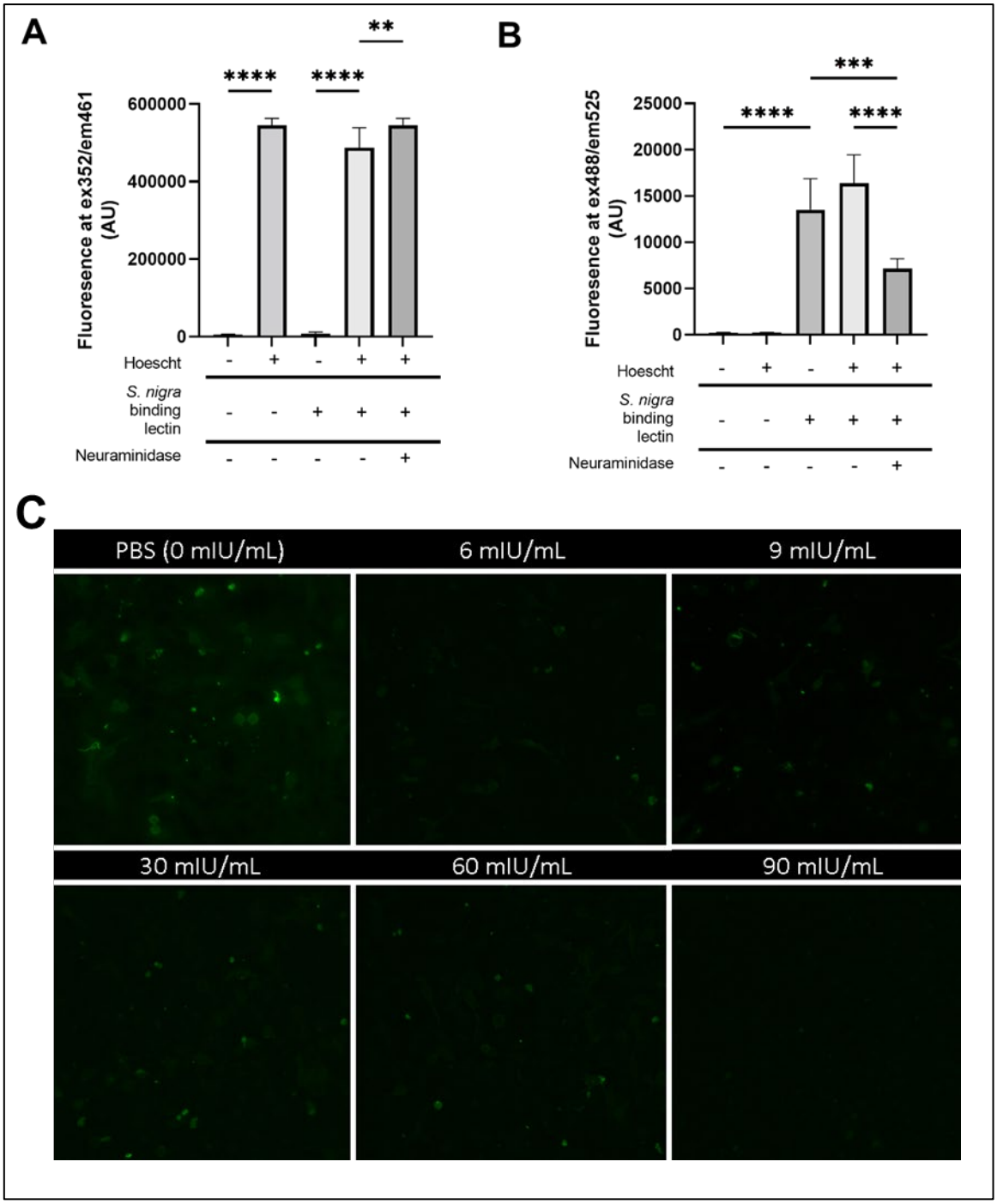

Figure IV-4. Successful validation of a novel sialic acid quantitation assay.

Hela were grown in black-walled, clear based 96-well plates for 24 hours and then treated with either **(A+B)** PBS or 90 mIU/mL *Clostridium perfingens* neuraminidase, or **(C)** PBS, 6, 9, 30, 60 and 90 mIU/mL, *C. perfingens* neuraminidase, and then fixed with 4% v/v PFA. **(A+B)** Cells were stained with combinations of either Hoechst-33342, *Sambucus nigra* sialic acid binding lectin conjugated to FITC or both. Fluorescence was measured for **(A)** Hoechst-33342 at ex_{352}/em_{461} and **(B)** FITC at ex_{488}/em_{525}. ** = $p<0.005$, *** = $p < 5x10^{-3}$, **** = $p < 5x10^{-4}$. One-way ANOVA with Bonferroni corrections (n=8). **(C)** Cells were viewed by fluorescence microscopy using an IX-83 inverted microscope at (40x magnification) and processed using CellSens imaging software.

IV.2.5 Sulphated GAGs are elevated during DENV and recruit FH to the cell surface

Abundance of HS and sialic acid were defined in DENV-infected cells to investigate whether induced FH protein was binding these ligands on the cell membrane. Sulphated GAGs were quantitated by DMMB assay, demonstrating significantly less sulphated GAGs in the supernatant from DENV-infected cells and their lysates **(Figure IV-5A-B)**. The novel sialic acid binding lectin-based assay validated in **Section IV.2.4** was used to quantitate membrane-bound sialic acid and demonstrated no significant difference between uninfected and DENV-infected cells **(Figure IV-5C)**. Next, HS and sialic acids were cleaved from cells using heparinases (*Flavobacterium* heparinase I, III) or a sialidase treatment (*Clostridium perfingens* neuraminidase). Pilot studies validated that sialidase-only treatments removed sialic acid but caused no change in FH released from uninfected or DENV-infected cells **(Figure A.4)** and given that sialic acid levels were unchanged by DENV-infection, sialidase-only treatments were not pursued further. Heparinase treatment significantly increased the amount of FH in the supernatant for heparinase treated, DENV-infected, cells compared to heparinase treated, uninfected, cells and vehicle treated DENV-infected cells **(Figure IV-5D)**. Combined addition of heparinase and sialidase released significantly more FH from DENV-infected cells but not uninfected cells. This suggests a shift towards HS-dependent binding of FH to the cell membrane in DENV-infected cells **(Figure IV-5D)**.

Malondialdehyde (MDA) is also a ligand present on cell surfaces that will recruit FH and MDA quantitation was pursued using a thiobarbituric acid assay on DENV-infected cell lysates. However, despite successful validation of the thiobarbituric acid to detect MDA (between 60 μM – 1 mM) **(Figure A.5)**, the concentration of MDA in cell lysates was below the detectable limit of detection.

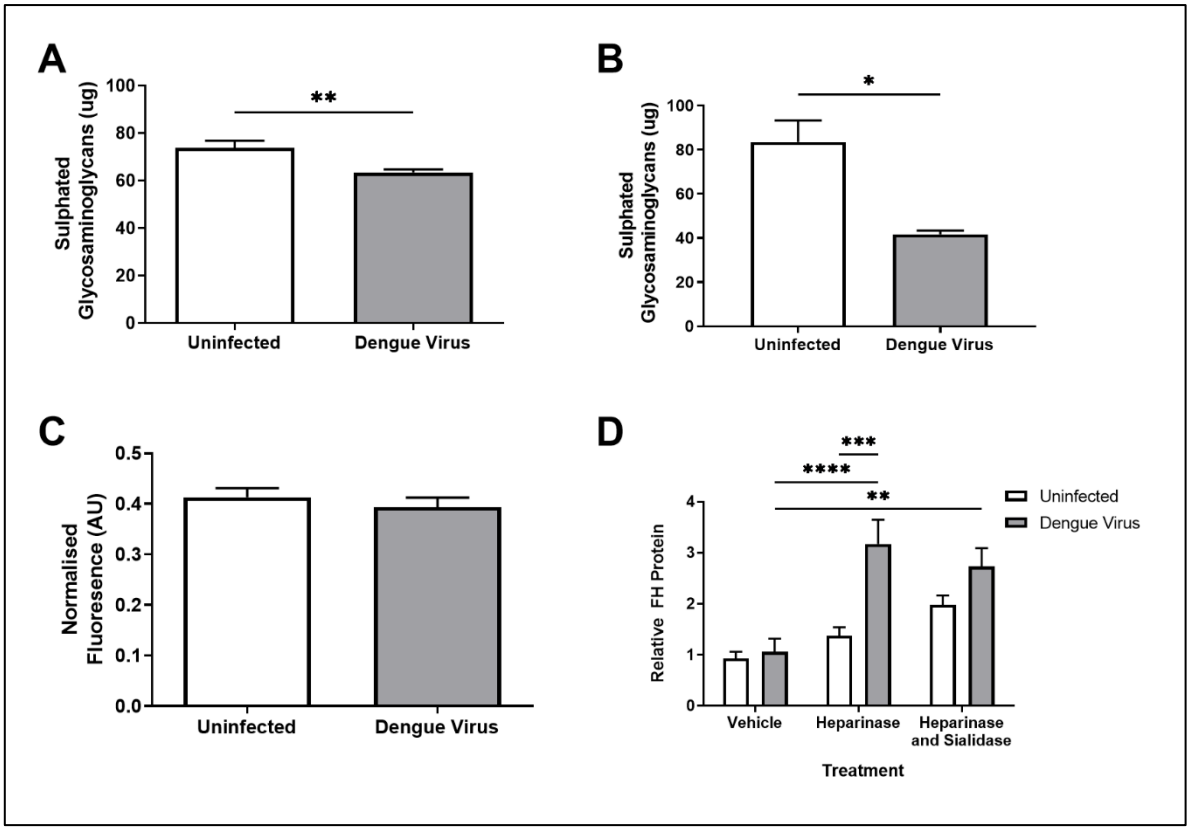

Figure IV-5 FH binding prioritises the sulphated glycoaminoglycans that are decreased in DENV-infected cells

HeLa were uninfected or DENV-infected and at 48 hpi **(A, B)** DMMB assay was used to quantify sulphated glycosaminoglycans **(A)** in the cell culture supernatant and **(B)** in cell lysates. **(A+B)** Results are representative of the mean ± standard error of three individual experiments in triplicate and analysed by Student's *t*-test. **(C)** Cells were fixed, and cell surface sialic acid detected with FITC-labelled *Sambucus nigra* binding lectin. Nuclei were stained with Hoechst-33342. Fluorescence (arbitrary units, AU) was quantitated with a fluorescence plate reader and values at ex_{352}/em_{461} normalised to ex_{488}/em_{525}. Results are representative of the means ± standard error of three separate experiments (n=44). Data were analysed by Student's *t*-test. **(D)** HeLa were uninfected or DENV-infected and at 48 hpi cells were treated with PBS + 5mM $CaCl_2$ (vehicle), heparinase I (400 µIU/mL) and III (heparinase) (415 µIU/mL) or heparinase and *Clostridium Perfingens* neuraminidase (90 mIU/mL) (heparinase and sialidase). After treatment, supernatant was collected, FH quantitated by ELISA and normalised to uninfected, vehicle controls. Results represent the pooled mean ± standard error from three independent experiments performed in triplicate and analysed by two-way ANOVA with Bonferroni corrections. FH = Factor H. **(A-D)** * = $p<0.05$, ** = $p<0.005$, *** = $p<5x10^{-3}$.

IV.2.6 FH localisation is altered by DENV-infection for both DENV-infected and bystander cells

Results from **Section IV.2.5** suggest altered GAGs on surface of DENV-infected cells and increased FH binding to these cell surface GAGs. Thus the cellular localisation of FH was investigated by IF staining and confocal microscopy. FH immunostaining in uninfected HeLa demonstrated low intensity fluorescence in a diffuse pattern across the cell **(Figure IV-6A)**. In contrast, FH staining in the DENV-challenged cells demonstrated fluorescence suggestive of localisation to the cell membrane, and high intensity fluorescence in the perinuclear zone around the nucleus, but not within it, and intracellularly, along cell networks. This distribution was observed in both dsRNA positive cells (undergoing active DENV replication), and in dsRNA negative cells of the DENV-challenged culture **(Figure IV-6B).**

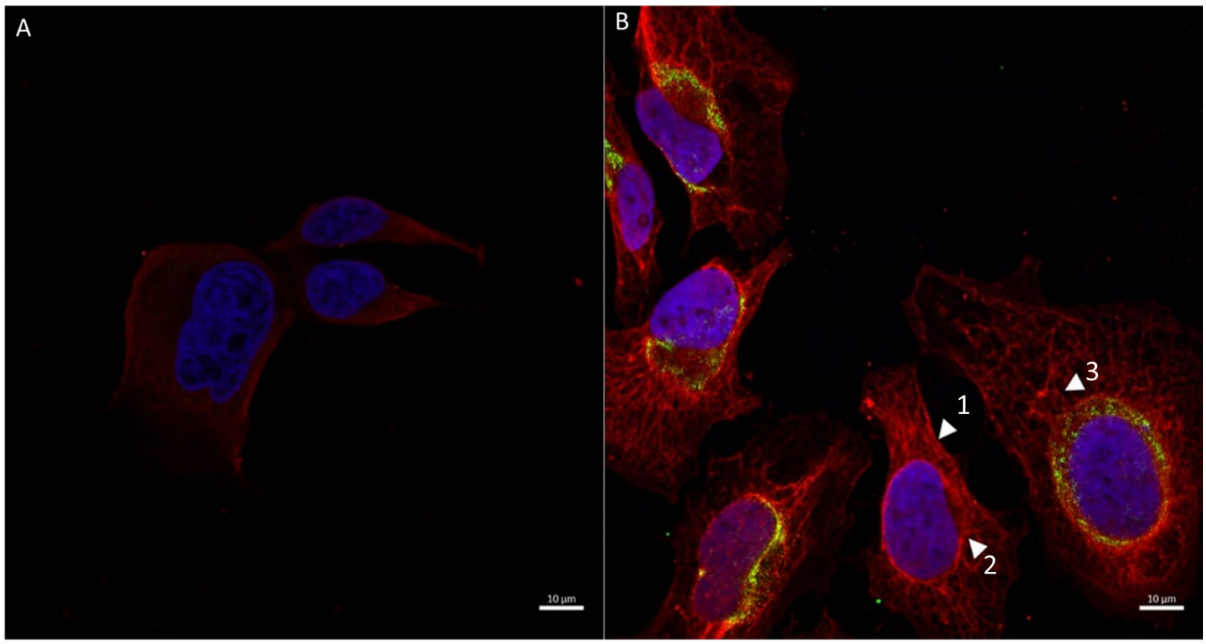

Figure IV-6 Cell associated FH is increased in DENV-infected cells and demonstrates altered cellular localisation (Still image of video file).

HeLa were either **(A)** uninfected or **(B)** DENV-infected and at 48 hpi cells were fixed, and immunostained for FH (Red) and dsRNA (Green), respectively. Nuclei were stained with Hoechst-33342 (Blue). In DENV-infected cells, (Arrow B1) FH displays patterns that suggest it is associated with the cell membrane and (Arrow B2) surrounds the nucleus in bystander and DENV-infected cells. (Arrow B3) FH-intracellular network associations were also observed. Cells were imaged by confocal microscopy and MP4 generated using Imaris image analysis software. Images were captured at 60x magnification using a Zeiss LSM880 confocal microscope, processed using Zen 3.0 software and are representative of 3 independent experiments. Fluorescence intensity between treatments were equalised simultaneously and are directly comparable.

Video file available at https://youtu.be/C7wO1RE_wdw and https://youtu.be/mjwsZh8lshU for uninfected and DENV-infected cells, respectively.

IV.3 Discussion

Dengue disease severity has been previously correlated with increased complement activity and decreased FH in patient blood.[217] Our laboratory has demonstrated that DENV infection in MDM induces cellular mRNA for both FB and FH but results in increased complement AP activity in cell supernatants due to an increase in secreted FB but not FH.[44] Here, a stable cell line that recapitulated increased FH mRNA but unchanged secreted protein was sought to facilitate investigations into the mechanisms of this phenomenon further. Following characterisation of DENV RNA production by four infected cell lines, it was observed HEK293 DENV RNA was markedly low. This may be due to high cytopathic effect, causing reduced viability of infected cells, which has previously been reported for DENV-2 infected HEK293 at 48 hpi.[333] The pro-monocytic cell line, U937 as well as HEK293 produced no detectable FH mRNA or protein and were not considered further. HeLa reproduced the same phenomena observed in primary MDM, with increased FH mRNA but unchanged FH secretion and thus, HeLa were selected as a model to focus on the molecular and intracellular dynamics of FH dysregulation during DENV infection. HeLa demonstrated changes in FH analogous to macrophages with respect to mRNA and extracellular protein. As HeLa do not represent the cell types DENV typically infects *in vivo*, these results should be validated in primary macrophages Surprisingly and in contrast to HeLa and primary macrophages, DENV-infection of Huh7.5 cells resulted in decreased FH mRNA and protein. The primary producer of circulating FH *in vivo* is the liver and although Huh7.5 cells are a hepatoma cell line; these cells are deficient in pathways that respond to type I interferons, which are known regulators of FH and thus not reflective of normal hepatocytes.[334] DENV is able to infect the liver *in vivo* such as in fatal-hepatitis induced by DENV.[335, 336] Additionally, the *CFH* promoter is predicted to contain a number of liver specific elements, such as the hepatic nuclear factor family identified in **Chapter III,** that drive constitutive FH expression. **Chapter III** and prior computational analysis of the *CFH* promoter from our laboratory has identified elements that suggest a strong dependency of the *CFH* promoter on interferon stimulated response elements.[43, 176, 337] Thus, the difference in FH mRNA induction between Huh7.5 and HeLa (as shown in **Figure IV-1)** MDM and EC, may result from interferon deficiency of Huh7.5 or potentially reflect hepatic response to DENV infection or true differences in FH regulation between MDM, EC and the liver.[44,

[334] The decreased FH production by Huh7.5, however, mimics the observed decreased circulating FH in severe dengue patients[217] and hence this is of interest to investigate further in the future. Further investigations to delineate the relationship between liver cell FH production and DENV-infection could involve other hepatic cell lines. This could be performed in other liver cell lines such as THLE2 or ideally primary liver cells if the challenges of acquiring primary liver cells can be overcome. HepG2 however should be avoided, as they do not produce detectable amounts of FH mRNA despite their hepatic origin.[338]

Our prior understanding of DENV effects on FH in MDM and EC, was extended further here, demonstrating that DENV-infected HeLa had increased cell-associated FH protein compared to uninfected cells, as quantitated by ELISA and IF staining. FH detected within lysates was immunoreactive but not able to be secreted, perhaps due to proteases or proteasomal degradation which was investigated using Western blotting and proteasome pathway inhibition. To investigate potential proteasomal degradation, DENV-infected cells were treated with two different proteasome inhibitors. Of note, there was an overall decrease in FH protein following MG132 treatment but not mRNA in both uninfected and DENV-infected cells. The mechanisms behind MG132-mediated reduction of FH protein, even in response to increased FH mRNA are unclear. Potentially, MG132 treatment interferes with pathways that facilitate FH secretion or promote internalisation of FH. Despite proteasome inhibitor treatment by either MG132 or bortezomib, FH mRNA was still induced by DENV, and FH protein release into the supernatant was unaffected, suggesting that the proteasome was not responsible for the reduced ability of infected cells to convert the induced FH mRNA into extracellular protein or the increase in FH immunoreactivity intracellularly. Future experiments that aim to investigate changes in FH secretion might benefit from the use of positive controls known to induce FH production and secretion such as type II interferons, LPS, PolyI:C or dexamethasone.[44, 183]

Consistent with the lack of identified proteolysis, Western blot confirmed the presence of the full-length 155 kDa FH species within and secreted by DENV-infected HeLa. Western blot, however, was not sensitive enough to discriminate the 2.5 fold change in FH quantitated by ELISA. Western blot also, on occasion, detected smaller FH-immunoreactive proteins of 50-55kDa. These products have the potential to represent

proteolytic products, FH isoforms or related proteins. However, our study did not detect FHL-1 or FHR-1 mRNA in DENV-infected HeLa, the most likely FH cross-reactive proteins based on homology.[197] Further, these 50-55kDa proteins were not reliably detected in all Western blot experiments nor were any FH isoforms detected by mass spectrometry in **Chapter V**. Thus, these 50-55 kDa proteins are likely to result from non-specific cross-reactivity. Importantly, the ELISA method has been validated and should not suffer similar non-specific issues since the monoclonal FH capture antibody does not cross react with FH isoforms or related proteins, and the sandwich design with a monoclonal FH detection antibody makes non-specific cross reactivity unlikely.[43]

Consistent with detection of full-length FH protein, the presence of full-length FH mRNA was demonstrated. The *CFH* gene transcripts can be alternatively spliced to produce either FH or FHL-1, a 42 kDa protein identical to FH_{SCR1-7} (Exons 1-9) of FH.[161] RT-qPCR with primers specific to the unique FHL-1 3' tail did not detect FHL-1 mRNA in DENV-infected HeLa. Further PCR analysis for the levels of transcripts from 3 sites across the full-length FH mRNA (representing detection of the FH 5', unique central and 3' transcript domains) demonstrated approximate equivalence, suggesting that the FH mRNA transcripts induced by DENV are not prematurely truncated and are likely full-length and able to translate full-length FH protein. Thus, it is unlikely that HeLa were producing a FH species other than full-length FH.

The increased cell-associated FH was expected to be via cell membrane sialic acid and HS binding, as these are the most important ligands for binding FH extracellularly to regulate C3 cleavage.[238, 259, 342] Alternatively, in response to oxidative stress, which is known to occur following DENV infection, cells can produce the FH ligand malondialdehyde (MDA) on the lipid membrane.[343, 344] The quantitation of MDA in cell culture lysates was explored by TBA assay initially, but the amount of MDA produced by HeLa in response to DENV was below the level of detection for the assay. In the future, the technique could be refined through implementation of high-performance liquid chromatography for more sensitive detection of MDA products.[345-347] As MDA was below the detectable limit of the assay, methods for quantitating the extracellular sialic acid and HS were sought. Current methods for detecting sialic acid specifically were time consuming and expensive when using commercial kits **(Table A.1)**. Thus, a fluorometric, novel sialic acid quantitation assay was developed for the purpose of

this project and has the potential for broader application. This is the first description of this method for sialic acid detection to my knowledge. Validation of the sialic acid assay was successful and presents an inexpensive assay for use on adherent cells *in vitro*. Although this analysis has not been refined for absolute sialic acid quantities, it is a useful tool for comparative quantitation of sialic acid with significantly reduced cost ($0.52 sample vs $6.70-$33.80 AUD per sample) and comparable time investment relative to existing methods **(Table A.1)**. Using this assay, DENV infection did not induce a significant change in cell surface sialic acid by 48 hpi, consistent with previous studies in EC.[15] DENV-infection decreased total cellular levels of sulphated GAGs concomitant with reduced supernatant GAGs. This is in support of prior reports demonstrating rapid shedding (within 1-4 hr) of GAGs from the glycocalyx of ECs in response to DENV-NS1 *in vitro*.[15, 259, 262] Additionally, our study has demonstrated an increase in the amount of FH that can be released from DENV-infected cells by cleavage of HS, but this was not further affected by concomitant sialidase treatment. This suggests that in an uninfected cell, cell surface FH is bound to both HS and sialic acid and potentially other ligands, but in DENV-infected cells, FH binding is largely via HS with less contribution of sialic acid, consistent with our finding of increased GAGs in DENV-infected cells. These data demonstrate dependence of FH-membrane binding on HS and sialic acid but could be further supported with confocal microscopy following heparinase and sialidase treatment. The consequences of increased GAG-FH binding on DENV-infected cells may be of benefit to the host. DENV requires cell-membrane interactions, including via HS to enter the cell and induction of extracellular HS or the shift to HS binding by FH may act to block DENV attachment and entry.[326] Following the observation that greater FH is released by heparinase treatment in DENV infected cells, total membrane-bound and cytosolic FH quantitation was attempted by fractional centrifugation of HeLa. The attempts to separate cellular components were unsuccessful due to unacceptably high concentration of the cytosolic protein IκBα in the nuclear fraction of cell lysates as examined by Western blot **(Figure A.6)** but could be reapproached with the use of commercial kits in the future.

Together these data suggest that intracellular re-localisation and retention of FH and extracellular FH recruitment by binding to GAG's following DENV infection may account for the discord between increased FH mRNA and unchanged FH secretion

from DENV-infected cells. At a cellular level, one potential consequence of this may be the blockade of further GAG-mediated DENV-infection, benefiting the host or reduced complement-mediated cell-killing, benefiting the virus. The effect of FH-GAG binding on viral replication could be tested by the pre-treatment of cells with exogenous FH and, for comparison, a heparan sulphate-binding molecule such as antithrombin.[348] After FH or antithrombin treatment, DENV-replication or intracellular viral protein could be measured by plaque assay or IF, respectively.

As cell fractionation was unsuccessful, confocal microscopy was used to visualise the localisation and distribution of the FH protein within DENV-infected cells. FH was in-part localised to the plasma membrane, as expected in both uninfected and DENV-infected cells. However, higher fluorescence intensity was observed for FH on the cell membrane and within DENV-infected HeLa compared to uninfected cells. This observation was supported by similar findings in a prior analysis of FH in DENV-infected MDM by flow cytometry.[44] Additionally, FH showed increased localisation at the perinuclear zone in the DENV-infected culture (DENV-infected and bystander). For both membrane and perinuclear associated FH, this increase in immunostaining and altered localisation was observed in cells of the DENV-challenged culture that were actively replicating DENV (dsRNA positive) and potentially non-productively infected or uninfected bystander cells (dsRNA negative), therefore FH induction can be induced *in trans*. This observation of induction of FH in both DENV-infected and uninfected bystander cells is also consistent with previous experiments in DENV-infected MDM that detected FH by flow cytometry on both infected and uninfected cells from the DENV-challenged culture, but interestingly this was not observed in DENV-infected EC.[44] This suggests that the DENV induction of FH may be indirect and secondary to release of soluble factors from macrophages and HeLa cells that are not present in EC. This observation lead to the investigation of soluble factors released from DENV-infected cells in **Chapter V**.

The finding of perinuclear FH localisation is novel and defining intracellular functions of FH during infection requires further investigation.[349] As described in **Chapter III,** (pp. 103) intracellular FH binds intracellular CTSL.[350] Here, FH directs cleavage of nascent C3 into C3b, which then generates and directs the opsonin iC3b to the extracellular membrane in apoptotic cells to reduce exposure of potential autoantigens to the adaptive immune system.[350] As an opsonin, iC3b decoration on the surface of

cells or DENV virions may promote phagocytosis of infectious viral particles.[349, 351] Importantly, existing patient data analysed in **Chapter III** revealed CTSL was one of the most highly increased transcripts investigated in whole blood cells. FH internalisation for nascent C3 cleavage may therefore be occurring for DENV-infected HeLa, although a simpler explanation is that FH is retained intracellularly following translation.[349, 352]

IV.4 Summary

This chapter has demonstrated that FH protein is produced in response to DENV infection, following successful FH mRNA transcription, although the protein is either not secreted or secreted protein binds cells and therefore, does not remain in fluid phase. In both DENV-infected and uninfected bystander cells, FH protein is increased and retained around the nucleus and along cell networks, with concomitant cell surface FH binding predominately via HS rather than sialic acid. The observation of increased FH protein in all cells of the DENV-challenged culture demonstrated that FH induction and perinuclear localisation could be induced in cells that were not replicating virus. Therefore the secreted agents that caused uniformly increased cell-associated FH were investigated in an attempt to identify candidates that might cause FH induction and altered localisation in **Chapter V**.

CHAPTER V

SECRETED MOLECULES FROM DENV INFECTED CELLS INDUCE FH mRNA AND CHANGES IN PROTEIN LOCALISATION OF FH TO THE CELL

V.1 Introduction

The observation in **Chapter IV** that DENV infection in HeLa was able to produce increased FH mRNA and cell-associated protein in both bystander and DENV infected cells demonstrated that some secreted factor was inducing these changes. The pathways and secreted proteins capable of inducing FH are largely uninvestigated, although early research has at least confirmed FH mRNA induction by IL-27 in mouse eyes and dexamethasone, IFN-γ, and retinoic acid *in vitro* using lung fibroblast cells (L929).[181, 183, 353, 354] Specifically, infection induced FH mRNA remains largely unexplored, although our laboratory has demonstrated that mimetics of viral RNA genomes (Poly I:C) strongly induce FH mRNA in MDM that results in increased secreted protein.[44] It was also demonstrated that mimicking bacterial infection with LPS significantly increased FH mRNA in MDM[44], which does not occur for LPS-treated L929, U937 or human skin fibroblasts *in vitro*.[355-357] There are many innate immune response pathways activated during infection predicted to induce FH by the *in silico* promoter analysis in **Chapter III**. Sites such as the NF-κB binding region have been confirmed to bind NF-κB *in vitro* through electromobility shift assays[182], however, no attempt has been made to induce FH mRNA through NF-κB activating stimuli specifically. Additionally, DENV-NS1 activates TLR-4, which is typically activated by LPS and causes NF-κB activation.[64] Therefore DENV-NS1 might induce FH mRNA induction through TLR-4 activation as in the case of LPS treated MDM. Evaluation of the induction of FH by secreted molecules during infection, such as DENV-NS1 and cytokines, was performed to further define complement activity and AP regulation during DENV-infection.

FH mRNA and intracellular protein localisation were induced in uninfected bystander cells, observed in **Chapter IV**. This led to the hypothesis that DENV-infected cells secrete agents that induce FH mRNA and cause resultant cell-associated FH for both DENV-infected and bystander cells. Therefore, the supernatants from DENV-infected HeLa were used to stimulate DENV-naïve HeLa and the acute effects of this treatment on FH mRNA induction and FH-cell association were assessed. The secreted proteins produced by DENV-infected HeLa were separated by molecular weight-based filtration and subject to mass spectrometry in an attempt to identify the candidates responsible for FH mRNA induction and cell-association in HeLa.

V.2 Results

V.2.1 The large proteins secreted by DENV infection acutely induce complement AP proteins

Cells were treated with unconditioned media, or infected with DENV (MOI=1) as controls or stimulated with DENV-infected HeLa supernatant was used to stimulate DENV-naïve HeLa and FH mRNA was quantitated by RT-qPCR. The supernatant of DENV-infected HeLa induced a dose-dependent trend of increased FH mRNA by 24 hpi and continued at 48 hpi **(Figure A.7).** To determine whether this effect was mediated by low molecular weight cytokines or larger secreted factors, the supernatant was fractionated into proteins above and below 50 kDa in size. Preparation of the supernatants for these experiments is described in **(Section II.15-16).** Cells were either treated with controls or supernatants of DENV-infected HeLa: supernatant from uninfected HeLa (uninfected control referred to as conditioned media hereafter) were used for uninfected controls, complete media containing DENV (MOI=1) (infected control referred to as DENV (MOI=1) hereafter) were used as DENV-infected controls, unfiltered supernatant, the >50 kDa or <50 kDa supernatant fractions. 50 kDa Molecular weight cut-off was selected as the most suitable molecular weight separation. This was due to separation of most cytokines (<50 kDa) from DENV virions and NS1 multimers (>50 kDa) in a commercially available size-based filtration system. Following stimulation, cellular RNA was harvested at 8 hpi for RT-qPCR. An 8 hpi incubation time was selected so that results might reflect response to stimuli rather than response to replicating virus, based on prior evidence in DENV-infected C6/36 and HEK293 cells.[333, 358]

Results demonstrated that FH mRNA was induced and significantly higher in HeLa treated with the >50 kDa supernatant fraction compared to conditioned media and DENV (MOI=1) but was not different for all other treatments **(Figure V-1A)**. FB and C3 are subunits of complexes that amplify the AP, previously shown to increase in MDM and EC infected with DENV at a protein level and so, were also investigated.[44] FB mRNA was increased in HeLa treated with the unfiltered and >50 kDa supernatant fraction **(Figure V-1B)**. C3 was not significantly different, despite an apparent trend in increased mRNA by >50 kDa supernatant fraction treated cells **(Figure V-1C)**. Although at 8 hpi, DENV replication from a new round of infection is expected to be low, DENV RNA was quantitated. Compared to DENV (MOI=1), the unfiltered

supernatant treated cells contained approximately 10-fold higher DENV RNA, the >50 kDa fractionated supernatant was not different. Additionally, the <50 kDa fraction yielded DENV RNA below the detectable limit of the qPCR **(Figure V-1D)**.

As DENV infection had previously been demonstrated to cause increased cell associated FH by IF microscopy and ELISA in **Chapter IV**, IF was repeated with cells also treated by the unfiltered, >50 kDa and <50 kDa fractionated supernatants at 24 hpi. RNA was previously collected at 8 hpi, before multiple rounds of DENV infection could occur. However, 24 hpi was selected for IF to provide time for protein translation and protein accumulation to produce levels detectable by IF. DENV (MOI=1) cells had the highest number of dsRNA positive cells, followed by unfiltered supernatant. However, dsRNA positive cells treated with unfiltered supernatant were observed less frequently, and dsRNA positive, >50 kDa treated cells were rare. No dsRNA positive cells were observed for any conditioned media or <50 kDa fractionated supernatant treated cells, the latter confirming fractionation successfully filtered infectious DENV particles **(Figure V-2)**.

Perinuclear FH was observed to occur for DENV-infected cells, >50 kDa and <50 kDa stimulated cells but not by cells stimulated with conditioned media or unfiltered supernatant. DENV (MOI=1) stimulated cells appeared to have higher intensity fluorescence for FH, as previously observed in **Section IV.2.1**. Cell-associated FH intensity appeared lowest in conditioned media-treated cells, comparable to the unfiltered supernatant. HeLa treated with >50 kDa supernatant appeared to have slightly stronger fluorescence relative to conditioned media controls. However, DENV-infected controls and the small <50 kDa fractionated supernatant appeared the highest intensity of cell associated FH **(Figure V-2)**.

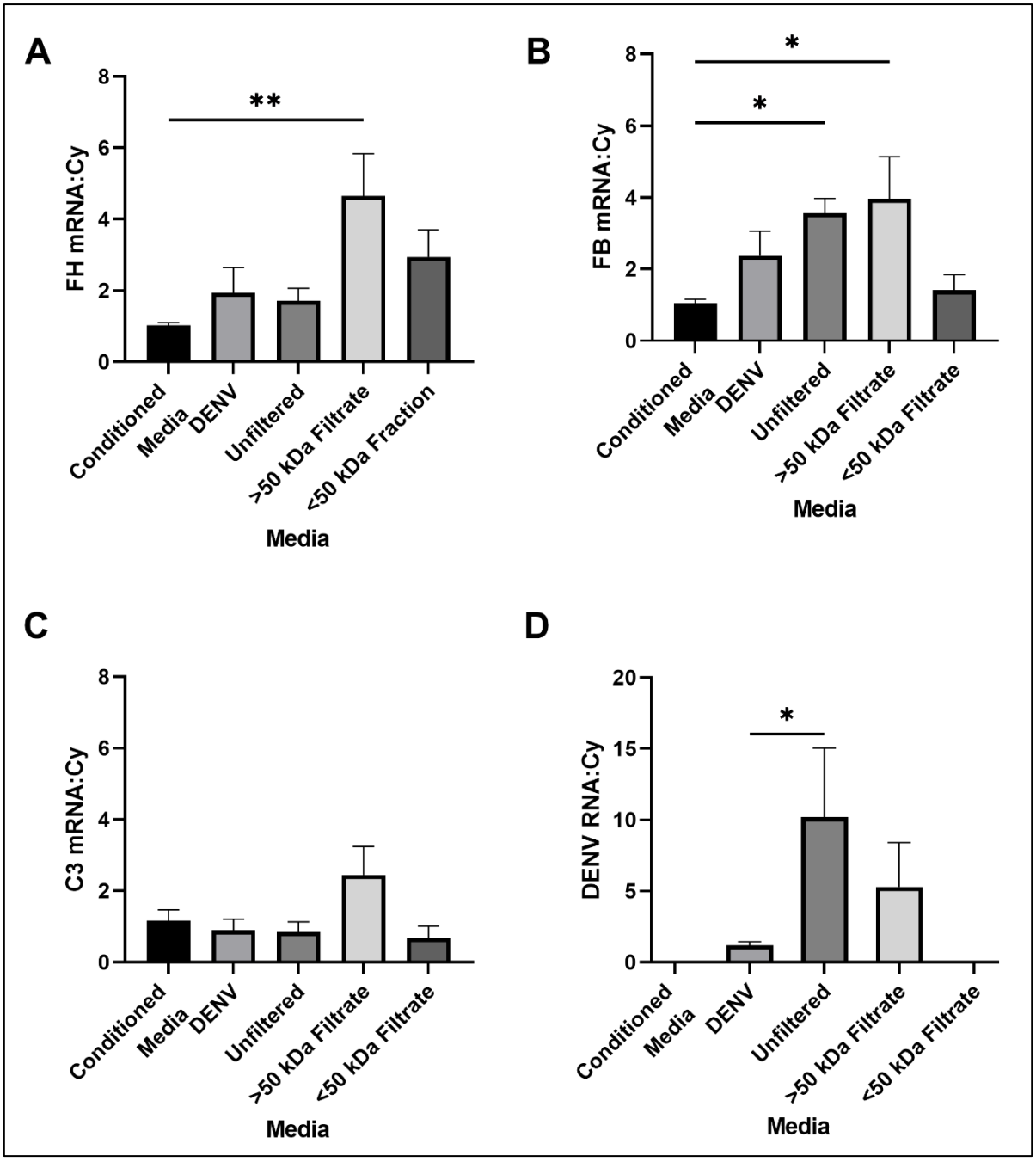

Figure V-1 Large, secreted molecules induce FH and FB mRNA within 8 hours.

(A-D) HeLa were treated with conditioned media, DENV (MOI=1), supernatant of DENV-infected HeLa, large (>50 kDa) or small (<50 kDa) proteins fractionated from the DENV-infected cell supernatant. RNA was then harvested 8 hpi for RT-qPCR for **(A)** FH **(B)** FB **(C)** C3 and **(D)** DENV. Ct values were normalised to cyclophilin and expressed relative to **(A-C)** conditioned media controls **(D)** DENV (MOI=1). Results represent the pooled mean ± standard error from 3 independent experiments performed in triplicate and analysed one-way ANOVA with Bonferroni corrections. *= $p<0.05$ ** = $p<0.005$.

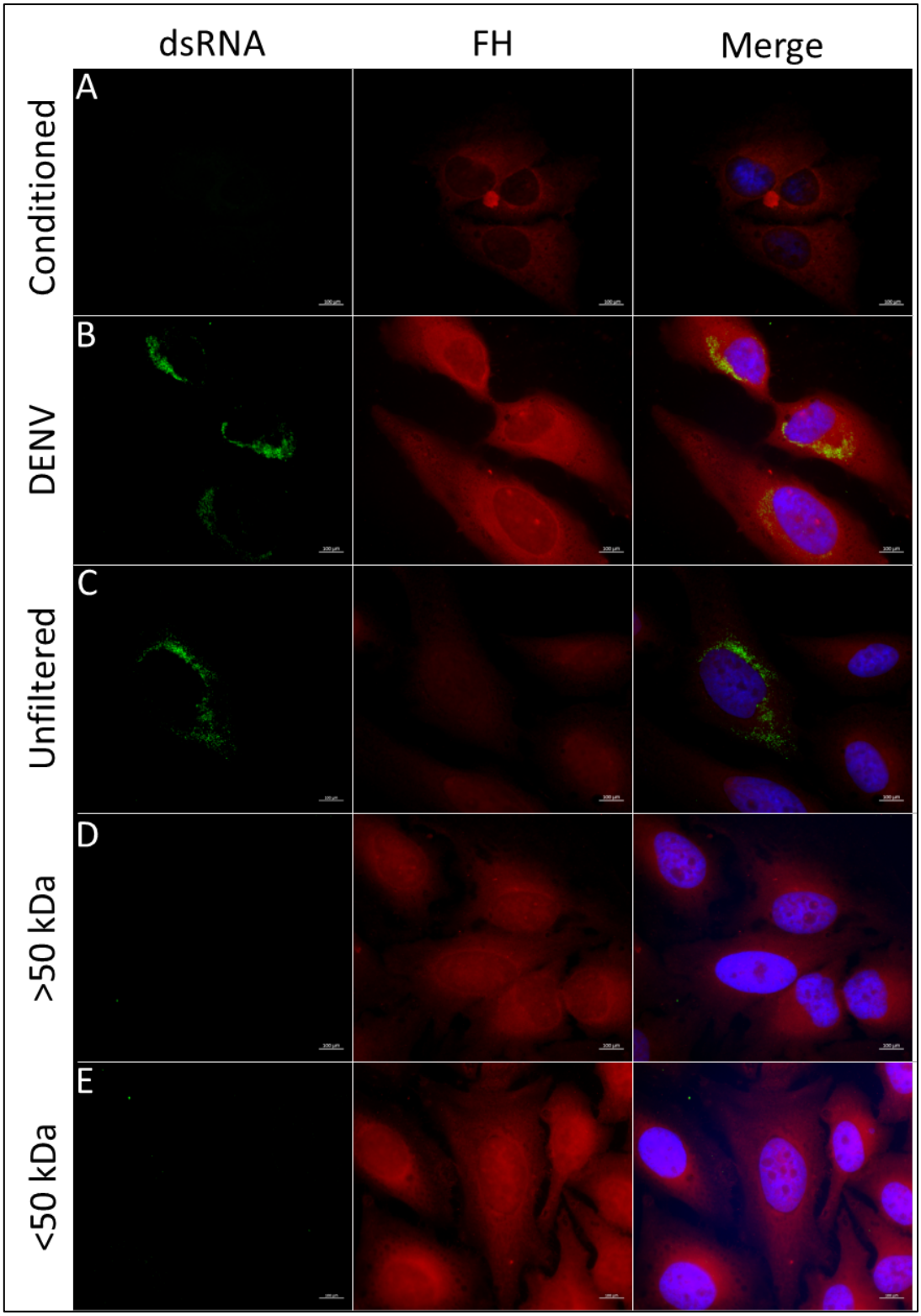

Figure V-2 Induction of FH protein-cell localisation can be induced by small and large secreted proteins

HeLa were treated with **(A)** conditioned media, **(B)** DENV (MOI=1), **(C)** supernatant of DENV-infected HeLa, **(D)** large (>50 kDa) or **(E)** small (<50 kDa) proteins fractionated from the DENV-infected cell supernatant. After 24 hours of infection or treatment, cells were fixed and stained for nuclei (blue), dsRNA (green) and FH (red). Images were captured with an Olympus AX70 fluorescence microscope at 100x magnification under oil immersion and processed using ZEN 3.0 (Blue) software, fluorescence intensity across images is directly comparable. Images are representative of three independent experiments. Scale bars represent 10 μM. DENV= Dengue Virus, dsRNA = double stranded RNA, FH = Factor H.

V.2.2 AP activating proteins C5, and FB were uniquely detected in the supernatant of DENV infected HeLa

As observed in **Section V.2.1**, FH mRNA abundance and protein localisation are influenced by the proteins secreted by DENV infected cells. Therefore, changes in the HeLa secretome following DENV infection were investigated. The presence and abundance of secreted and extracellular proteins uniquely expressed following DENV infection were determined by mass spectrometry for qualitative analysis. Proteins detected in the DENV-infected cell supernatant conditioned media controls were compared in an attempt to identify the potential candidates to investigated FH mRNA induction by the >50 kDa fraction further. Detected proteins with low confidence reads (SUM PEP score <5) or low abundance proteins smaller than 45 kDa (below the lower threshold of the filtration system) were not considered. 84 proteins assigned to either secreted or extracellular localisation by the Thermo Proteome Discoverer software were detected within the >50 kDa supernatant fraction **(Table V-1)** and 129 secreted or extracellular proteins in the conditioned media were detected by mass spectrometry. The large >50 kDa fraction of the DENV-infected HeLa supernatant contained all of the DENV proteins found extracellularly: capsid, membrane, envelope, likely as part of an assembled virion, and NS1, as expected **(Table V-2)**. Of the 84 secreted and extracellular host-proteins, 9 were detectable in the >50 kDa supernatant fraction that were not in the conditioned media. Interestingly, two complement cascade components, C5 and FB were amongst these 9 unique proteins. **(Table V-2)**. Many large complement components were also detectable within conditioned media, unfiltered and >50 kDa supernatant (CD109, C1s, C1r, C1q, C3, C4, C7, C9, FH) (data available at https://zenodo.org/record/6911339#.YuDXjHZByUl).

Proteins with >Log2 fold-changes were recorded for descriptive purposes but were unable to be compared for statistical analysis and differences are therefore qualitative only. Although many proteins were not uniquely present in the DENV-infected supernatant, some of the most abundant proteins within the >50 kDa sample were identified as complement proteins or proteins that facilitate DENV infection, these were Inter-Alpha-Trypsin Inhibitor Heavy Chain 2 (ITIH2), α2-Macroglobulin (α_2M) and complement C4 **(Table V-2)**. Comparison of conditioned media and the >50 kDa fraction revealed α2M was one of the most abundant proteins (2^{nd} percentile) in the >50 kDa fraction but far lower in the ranked abundance of proteins in the conditioned media (27^{th} percentile). Similarly, ITIH2 (3^{rd} percentile) and C4 (34^{th} percentile) were

abundant in the >50kDa fraction but either low or approaching the lower limit of detection in the conditioned media (47th and 88th Percentiles respectively). FH was detected in the conditioned media (26th percentile) and >50 kDa (50th percentile), which showed secreted FH had lower ranked abundance in fractionated supernatant of DENV-infected HeLa. Similar trends in protein abundance for unfiltered supernatant compared to conditioned media were observed, demonstrating that these differences are unlikely a product of fractionation itself (data available at https://zenodo.org/record/6911339#.YuDXjHZByUl).

Table V-1 Large secreted and extracellular proteins released from DENV-infected HeLa, as detected by mass spectrometry.
All secreted and extracellular proteins present in the >50kDa fraction of supernatant from DENV-infected HeLa, as detected by mass spectrometry that had summary peptide scores >5 with molecular weight >45 kDa. Proteins listed from highest to lowest abundance. Proteins with additional notes have been highlighted.

Gene Name	Abundance	Peptide score	Molecular Weight (kDa)	Protein Identified
LTF	3.24E+09	6.979	78.3	Lactotransferrin
A2M	1.49E+09	44.999	163.2	Alpha-2-macroglobulin
ITIH2	1.09E+09	30.269	106.4	Inter-alpha-trypsin inhibitor heavy chain H2
TGFBI	7.04E+08	173.209	74.6	Transforming growth factor-beta-induced protein ig-h3 (Fragment)
AFP	4.80E+08	29.035	50.7	Alpha Fetoprotein
PKM	3.96E+08	186.302	57.9	Pyruvate kinase
HSP90AB1	3.68E+08	89.436	83.2	Heat shock protein 90kDa alpha (Cytosolic)
LGALS3BP	3.10E+08	38.533	64.1	Galectin-3-binding protein
C3*	1.93E+08	98.433	187	Complement C3
GSN	1.84E+08	64.172	78.8	Actin-depolymerizing factor
HSPA8	1.57E+08	104.541	70.9	Epididymis luminal protein 33
FLNA	1.24E+08	143.299	280.6	Filamin-A
NS1	1.17E+08	31.45	379.5	Dengue Virus Non-structural 1
DENV-C**	1.17E+08	31.45	13	DENV Capsid Protein
FBLN1	1.17E+08	21.995	70.5	Fibulin-1
COL3A1	8.96E+07	44.107	138.5	Collagen alpha-1(III) chain
FN1	8.86E+07	72.794	259	Fibronectin
SERPINF1	8.70E+07	15.602	46.3	Pigment epithelium-derived factor
THBS1	7.75E+07	51.202	129.3	Thrombospondin-1
PXDN	7.55E+07	74.294	165.2	Peroxidasin homolog
VCP	7.52E+07	76.46	89.3	Transitional endoplasmic reticulum ATPase
QSOX1	7.51E+07	29.524	82.5	Sulfhydryl oxidase 1
APOB	7.34E+07	31.91	489.5	Mutant Apo B 100
EEF2	7.10E+07	40.614	95.3	Elongation factor 2
DENV M	6.78E+07	8	379.3	DENV Membrane Protein
PCSK9	6.21E+07	69.239	74.2	Proprotein convertase subtilisin
HSP90AA1	5.98E+07	96.581	84.6	Epididymis luminal secretory protein 52
MMP2	5.39E+07	15.609	73.8	72 kDa gelatinase
EEF1A1	5.26E+07	10.396	50.2	Elongation factor 1-alpha (Fragment)
C4A	5.04E+07	7.954	187.6	Complement C4
HSPG2	4.93E+07	75.478	463.7	Heparan sulphate proteoglycan 2 (Perlecan)
HEXB	4.51E+07	27.858	63.1	Beta-hexosaminidase subunit beta
HGFAC	4.17E+07	12.805	71.4	Hepatocyte growth factor activator
C5	3.71E+07	12.32	188.2	Complement C5
COL12A1	3.39E+07	70.508	332.9	Collagen alpha-1(XII) chain
SMOC1	3.06E+07	13.034	48.1	SPARC-related modular calcium-binding protein 1
LAMC1	2.82E+07	45.91	177.5	Laminin subunit gamma-1
COL5A1	2.53E+07	54.097	183.4	Collagen
TUBB4B	2.48E+07	39.089	49.8	Tubulin beta chain
ACLY	2.47E+07	42.4	120.8	ATP-citrate synthase
TLN1	2.43E+07	78.196	269.6	Talin-1
DAG1	2.35E+07	23.289	97.5	Alpha-dystroglycan

LAMB1	2.21E+07	43.336	197.9	Laminin subunit beta-1
CFH	1.60E+07	19.189	139	Complement Factor H
VCL	1.57E+07	51.601	116.6	Metavinculin
TUBB	1.49E+07	46.982	46.5	Tubulin beta chain
VASN	1.46E+07	17.343	71.7	Vasorin
DENV E**	1.44E+07	3.634	54.2	Envelope protein E (Fragment)
FUCA2	1.28E+07	18.991	54	Plasma alpha-L-fucosidase
LAMA4	1.15E+07	34.603	201.7	Laminin, alpha 4
COL1A1	1.09E+07	19.886	84.7	Collagen
BMP1	1.07E+07	9.205	90.6	Metalloendopeptidase (Fragment)
CCT2	1.06E+07	32.074	57.5	CCT-beta
CCT8	9.91E+06	18.367	59.6	T-complex protein 1 subunit theta
GPI	9.70E+06	18.577	63.1	Glucose-6-phosphate isomerase
LAMA5	8.73E+06	27.154	399.5	Laminin subunit alpha-5
EFEMP1	8.48E+06	8.903	45.7	EGF containing fibulin-like extracellular matrix protein 1
KPNB1	8.13E+06	17.288	97.1	Importin subunit beta-1
COL2A1	7.81E+06	7.757	141.7	Collagen alpha-1(II) chain
P4HB	7.61E+06	25.263	55.3	Protein disulphide-isomerase
FBN1	7.02E+06	14.559	312.1	Fibrillin-1
PCOLCE	6.98E+06	11.626	47.9	Procollagen C-endopeptidase enhancer
COMP	6.80E+06	13.269	82.7	Cartilage oligomeric matrix protein variant (Fragment)
FSTL4	6.71E+06	25.644	93	Follistatin-related protein 4
GDI2	6.63E+06	23.879	51.1	Rab GDP dissociation inhibitor
IDH1	6.32E+06	12.998	46.6	Isocitrate dehydrogenase [NADP] cytoplasmic
LPL	6.02E+06	16.614	51.7	Lipoprotein lipase
XRCC5	5.42E+06	9.233	82.7	X-ray repair cross-complementing protein 5
XRCC6	5.22E+06	21.869	69.7	ATP-dependent DNA helicase 2 subunit 1
MYH9	5.03E+06	27.078	226.4	Non-muscle myosin heavy chain 9
CAND1	3.83E+06	10.034	85.4	Cullin-associated NEDD8-dissociated protein 1 (Fragment)
CPN1	3.79E+06	5.762	52.3	Carboxypeptidase N catalytic chain
COL7A1	3.79E+06	18.612	295	Collagen alpha-1(VII) chain
SERPINI1	3.33E+06	11.721	46.4	Serpin peptidase inhibitor clade I member 1 isoform 1 (Fragment)
PAM	3.29E+06	8.556	108.3	Peptidyl-glycine alpha-amidating monooxygenase
TFRC	2.80E+06	11.749	75.9	Transferrin receptor protein 1
IGSF10	2.76E+06	12.181	290.7	Immunoglobulin superfamily member 10
MUC16	2.47E+06	13.149	1518.2	Mucin-16
CAP1	2.33E+06	8.686	49	Adenylyl cyclase-associated protein 1
CDH13	2.12E+06	8.175	78.2	Cadherin-13
ILF3	1.57E+06	11.531	50.1	Interleukin enhancer binding factor 3 isoform c variant (Fragment)
CFB	1.02E+06	5.33	85.5	Complement Factor B
CD109	9.09E+05	13.917	161.6	CD109 antigen
JUP	8.49E+05	17.334	81.7	Junction plakoglobin (Fragment)

*C3 detection does not delineate whole C3 or the large C3b cleavage product

**DENV-C and DENV-E were likely detected as part of the assembled virion

Table V-2. Ranked abundance of proteins detected in the >50 kDa fraction of DENV-infected HeLa supernatant compared to uninfected HeLa cell culture supernatant (conditioned media). Differences expressed as Log2-fold change. Proteins highlighted in blue are members of the complement family and proteins highlighted in yellow are known to interact with DENV infection and replication.

	Gene Name	>50 kDa		Conditioned Media		Molecular Weight (kDa)	Protein Identified	Abundance in >50 kDa fraction	Log2-Fold change (>50 kDa:Conditioned Media)
		Ranked Abundance (Percentile)	Peptide score	Ranked Abundance (Percentile)	Peptide score				
Novel (Host)	**HBA2**	14(14%)	26.337	**ND**	**ND**	15.2	Mutant haemoglobin alpha 2 globin chain	1.54E+08	-
	APOB	26(26%)	31.91	**ND**	**ND**	489.5	Mutant Apo B 100	7.34E+07	-
	MMP2	32(32%)	15.609	**ND**	**ND**	73.8	72 kDa gelatinase	5.39E+07	-
	C5	40(40%)	12.32	**ND**	**ND**	188.2	Complement C5	3.71E+07	-
	COL2A1	68(67%)	7.757	**ND**	**ND**	141.7	Collagen alpha-1(II) chain	7.81E+06	-
	P4HB	69(68%)	25.263	**ND**	**ND**	55.3	Protein disulphide-isomerase	7.61E+06	-
	CPN1	83(82%)	5.762	**ND**	**ND**	52.3	Carboxypeptidase N catalytic chain	3.79E+06	-
	B4GALT1	89(88%)	39.789	**ND**	**ND**	43.9	Beta-1,4-galactosyltransferase 1	2.67E+06	-
	CFB	99(98%)	5.33	**ND**	**ND**	85.5	Complement Factor B	1.02E+06	-
DENV	**DENV E**	54(53%)	3.634	**ND**	**ND**	54.2	Envelope protein E (Fragment)	1.44E+07	-
	DENV M	28(28%)	8	**ND**	**ND**	379.3	DENV Membrane Protein	6.78E+07	-
	NS1	17(17%)	31.45	**ND**	**ND**	379.5	Dengue Virus Non-structural 1	1.17E+08	-
Increased Proteins	**ITIH2**	3(3%)	30.269	108(47%)	10.261	106.4	Inter-alpha-trypsin inhibitor heavy chain H2	1.09E+09	5.98
	C4A	34(34%)	7.954	204(88%)	6.553	187.6	Complement C4	5.04E+07	5.22
	A2M	2(2%)	44.999	62(27%)	97.315	163.2	Alpha-2-macroglobulin	1.49E+09	5.09
	TUBB	52(51%)	46.982	215(93%)	64.328	46.5	Tubulin beta chain	1.49E+07	4.06

	CAND1	82(81%)	10.034	225(97%)	12.185	85.4	Cullin-associated NEDD8-dissociated protein 1 (Fragment)	3.83E+06	3.01
	LUM	15(15%)	26.395	102(44%)	35.48	38.4	Lumican	1.35E+08	2.72
	TGFBI	4(4%)	173.209	30(13%)	181.499	74.6	Transforming growth factor-beta-induced protein ig-h3 (Fragment)	7.04E+08	2.25
	LPL	77(76%)	16.614	203(88%)	14.411	51.7	Lipoprotein lipase	6.02E+06	2.12
	HSP90AA1	31(31%)	96.581	116(50%)	94.731	84.6	Epididymis luminal secretory protein 52	5.98E+07	2.12
	LAMA5	65(64%)	27.154	193(84%)	15.808	399.5	Laminin subunit alpha-5	8.73E+06	2.00
	QSOX1	25(25%)	29.524	84(37%)	37.146	82.5	Sulfhydryl oxidase 1	7.51E+07	1.44
	PXDN	23(23%)	74.294	76(33%)	92.088	165.2	Peroxidasin homolog	7.55E+07	1.24
	BMP1	60(59%)	9.205	166(72%)	12.794	90.6	Metalloendopeptidase (Fragment)	1.07E+07	1.23
	C3	9(9%)	98.433	46(20%)	112.412	187	Complement C3	1.93E+08	1.17
Decreased Proteins	**CFH**	50(50%)	19.189	60(26%)	78.531	139	Complement Factor H	1.60E+07	-1.72
	GPI	63(62%)	18.577	5(3%)	329.868	63.1	Glucose-6-phosphate isomerase	9.70E+06	-1.89
	CD109	100(99%)	13.917	130(57%)	46.303	161.6	CD109 antigen	9.09E+05	-3.62
	MUC16	92(91%)	13.149	41(18%)	79.557	1518.2	Mucin-16	2.47E+06	-3.86
	JUP	101(100%)	17.334	85(37%)	62.74	81.7	Junction plakoglobin (Fragment)	8.49E+05	-5.4

V.2.3 Complement factor D, vitronectin, IL-6 and IL-8 are increased following DENV infection

As in **Section V.2.2**, mass spectrometry was performed to qualitatively determine the presence of secreted and extracellular proteins uniquely expressed following DENV infection in the <50 kDa fraction of proteins. Detected proteins with low confidence reads (SUM PEP score <5) and larger than 55 kDa (above the upper threshold of the filtration system) were excluded, many probably present as cleaved or degraded proteins. 152 secreted or extracellular proteins were detected within the <50 kDa supernatant fraction **(Table V-3)**. Mass spectrometry determined that the <50 kDa fraction of the DENV-infected supernatant contained only two complement proteins, Factor D (FD) (24.4 kDa) and vitronectin (54.3 kDa) as expected, as most complement proteins are larger than 100 kDa.[359]

Of the 152 secreted and extracellular host-proteins, 40 were uniquely detected in the <50 kDa supernatant fraction. The complement protein vitronectin and the sialidase NEU1, which was investigated in **Chapter III**, were uniquely detected within the <50 kDa supernatant fraction. Any relationship between the 38 other uniquely detected proteins and the complement cascade have not yet been documented in the literature.

The only complement or complement-interacting protein with a notable change was FD, which was ~2.2-fold lower in <50 kDa than in conditioned media. Increased arginase-1 (ARG1) relative abundance was also observed. Interestingly, dengue patient whole-blood (ARG1) mRNA abundance have been correlated to DENV severity.[272] In **Table V-4**, ARG1 shifted in abundance from the 54th to 21st percentile of proteins detected. Two forms of tubulin (TUBB and TUBA4A) experienced large shifts in ranked abundance (97th percentile to 8th percentile and 16th percentile to 4th percentile respectively) **(Table V-4)**. Protein abundance in the <50 kDa supernatant fraction were reflective of the abundance for the same proteins in the unfiltered supernatant. (Data available at https://zenodo.org/record/6911339#.YuDXjHZByUl).

No cytokine or chemokine was within the limit of detection for the <50 kDa fraction of the supernatant by mass spectrometry. However, a pilot analysis performed on the <50 kDa fraction by Bio-Plex array was able to detect IL-6 and IL-8. IL-6 was detectable in both conditioned and fractionated media (1.99 vs 18.23 pg/mL) and IL-8 was detectable in <50 kDa supernatant fraction (1.39 pg/mL) but not conditioned

media controls. By Bio-Plex cytokine array analysis, IFN-γ, GM-CSF, TNF-α, IL-2, IL-4, IL-10 were below the limit of detection for both conditioned media and the <50 kDa supernatant fraction **(Figure V-3)**. Thus, mass spectrometry was not sensitive enough to detect IL-6 and IL-8 that were later confirmed to be present by cytokine array.

Table V-3. Small secreted and extracellular proteins released from DENV-infected HeLa, as detected by mass spectrometry.

All secreted and extracellular proteins present in the <50kDa fraction of supernatant from DENV-infected HeLa, as detected by mass spectrometry that had summary peptide scores >5 and molecular weight below 55 kDa (upper limit of filtration size). Proteins listed from highest to lowest abundance.

Gene Name	Abundance	Peptide score	Molecular Weight (kDa)	Protein Identified
ALDOA	2.47E+09	378.2	39.4	Fructose-bisphosphate aldolase
PPIA	2.25E+09	261.2	18	Peptidyl-prolyl cis-trans isomerase A
ANXA2	1.35E+09	329.3	38.6	Annexin A2
HIST1H4A	9.89E+08	93.4	11.4	Histone H4
EEF1A1	7.68E+08	177.9	50.2	Elongation factor 1-alpha (Fragment)
TUBA4A	7.43E+08	281.2	49.9	Tubulin alpha-4A chain
GDI2	6.50E+08	366.0	50.6	Rab GDP dissociation inhibitor beta
ANXA5	6.02E+08	162.4	35.9	Annexin A5
HNRNPA2B1	5.69E+08	187.2	37.4	Heterogeneous nuclear ribonucleoproteins A2/B1
GSTP1	4.69E+08	220.8	23.3	GST class-pi (Fragment)
IGFBP7	4.65E+08	105.3	29.1	Insulin-like growth factor-binding protein 7
ANXA1	4.26E+08	152.7	38.7	Annexin A1
TUBB	3.89E+08	194.5	47.7	Tubulin beta chain (Fragment)
IDH1	3.69E+08	210.6	46.6	Isocitrate dehydrogenase [NADP] cytoplasmic
AFP	3.69E+08	35.5	50.7	cDNA FLJ57154, highly similar to Alpha-fetoprotein
PGAM1	3.38E+08	234.3	28.8	Phosphoglycerate mutase
B2M	3.34E+08	143.1	13.9	Beta-2-microglobulin
FSTL1	3.09E+08	84.4	35	Follistatin-related protein 1
PNP	2.67E+08	134.6	32.5	Purine nucleoside phosphorylase
TIMP1	2.56E+08	46.4	23.2	Metalloproteinase inhibitor 1
SERPINB1	2.26E+08	110.1	42.7	Leukocyte elastase inhibitor
ALDOC	1.99E+08	156.4	39.4	Fructose-bisphosphate aldolase C
PA2G4	1.81E+08	137.1	43.8	Proliferation-associated 2G4
PRDX6	1.79E+08	131.0	25	Peroxiredoxin-6
GGH	1.78E+08	78.7	35.9	Gamma-glutamyl hydrolase
SERPINB6	1.76E+08	165.6	42.6	Serpin B6
AKR1B1	1.63E+08	96.2	35.8	Aldo-keto reductase family 1 member B1
CALU	1.55E+08	144.3	37.1	Calumenin
HNRNPC	1.47E+08	62.9	32	Heterogeneous nuclear ribonucleoproteins C1/C2
CST3	1.33E+08	38.0	15.8	Epididymis secretory protein Li 2
FABP5	1.27E+08	50.2	15.2	Fatty acid-binding protein 5
ARG1	1.21E+08	37.6	34.7	Arginase-1
PSMD6	1.13E+08	48.5	45.5	26S proteasome non-ATPase regulatory subunit 6
SFN	1.08E+08	113.3	27.8	Stratifin
CALM3	1.03E+08	106.0	16.8	Calmodulin-3
S100A4	1.01E+08	17.2	11.7	S100 Calcium Binding Protein A4

PDAP1	1.01E+08	41.4	20.6	28 kDa heat- and acid-stable phosphoprotein
HBA2	9.17E+07	31.4	15.2	Haemoglobin subunit alpha
SOD1	8.82E+07	123.9	15.9	Superoxide dismutase [Cu-Zn]
TXN	8.63E+07	36.1	11.7	Thioredoxin
H3F3A	8.46E+07	9.0	15.3	Histone H3.3
SMOC1	7.85E+07	68.0	48.1	SPARC-related modular calcium-binding protein 1
PSMB1	7.37E+07	43.9	26.5	Proteasome subunit beta type-1
MYDGF	7.30E+07	30.4	18.8	Myeloid-derived growth factor
SERPINB5	7.27E+07	63.0	42.1	Serpin B5
PRDX4	6.36E+07	97.1	30.5	Peroxiredoxin-4
SERPINF1	6.22E+07	31.7	46.3	Pigment epithelium-derived factor
CTSB	6.06E+07	69.8	37.8	Cathepsin B
NPC2	5.67E+07	44.2	19.2	Epididymal secretory protein E1
MANF	5.66E+07	74.5	20.7	Mesencephalic astrocyte-derived neurotrophic factor
CTSL	5.52E+07	25.1	37.5	Cathepsin L
PCOLCE	5.28E+07	70.8	47.9	Procollagen C-endopeptidase enhancer
PSMA5	5.25E+07	43.5	26.4	Proteasome subunit alpha type
ILF2	5.23E+07	84.0	38.9	Interleukin enhancer-binding factor 2
CAP1	5.07E+07	97.7	51.6	Adenylyl cyclase-associated protein 1
TIMP2	4.96E+07	56.6	20.2	Metalloproteinase inhibitor 2
PDXK	4.74E+07	37.1	30.6	Pyridoxal kinase
CAPZA1	4.67E+07	50.3	32.9	F-actin-capping protein subunit alpha-1
TMSB4X	4.21E+07	30.4	5.1	Thymosin beta-4
ACTR2	4.05E+07	77.2	44.7	Actin-related protein 2
SERPINE1	3.86E+07	57.0	45	Serpin peptidase inhibitor, clade E (Nexin, plasminogen activator inhibitor type 1), member 1
PSMC2	3.69E+07	58.9	48.6	26S proteasome AAA-ATPase subunit RPT1
FUCA2	3.60E+07	34.4	54	Plasma alpha-L-fucosidase
HMGB1	3.53E+07	21.3	18.3	High mobility group protein B1
TAGLN2	3.40E+07	81.5	22.4	Transgelin-2
APRT	3.37E+07	28.0	19.6	Adenine phosphoribosyltransferase
PSMD12	3.30E+07	10.7	52.9	26S proteasome non-ATPase regulatory subunit 12
CTSC	3.29E+07	59.2	50.1	Cathepsin C
PSMD11	3.11E+07	48.2	47.4	26S proteasome non-ATPase regulatory subunit 11
EFEMP1	3.07E+07	37.4	45.7	EGF containing fibulin-like extracellular matrix protein 1 isoform 2 (Fragment)
LUM	3.03E+07	28.2	38.4	Lumican
OLA1	3.01E+07	38.6	44.7	Obg-like ATPase 1
SERPINE2	2.95E+07	57.2	44	Serpin Family E Member 2
CYB5R3	2.50E+07	44.9	33.2	NADH-cytochrome b5 reductase
NIT2	2.47E+07	47.0	30.6	Omega-amidase NIT2
SERPINI1	2.43E+07	31.3	46.4	Serpin peptidase inhibitor clade I member 1 isoform 1 (Fragment)
SPOCK1	2.09E+07	31.2	49.1	Testican-1

DPP7	2.05E+07	26.5	54.3	Dipeptidyl peptidase 2
CALR	2.04E+07	54.1	46.9	Calreticulin variant (Fragment)
PPIE	1.91E+07	33.1	24.1	Peptidyl-prolyl cis-trans isomerase
B4GALT1	1.83E+07	69.3	43.9	Beta-1,4-galactosyltransferase 1
AIMP1	1.80E+07	46.2	34.3	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1
DCD	1.75E+07	22.9	11.3	Dermcidin
VAT1	1.67E+07	25.7	41.9	Vesicle amine transport protein 1 homolog (T californica)
FUCA1	1.52E+07	22.7	53.7	Tissue alpha-L-fucosidase
MIF	1.44E+07	14.8	12.5	Epididymis secretory sperm binding protein (Fragment)
HEBP2	1.38E+07	6.8	24	HEBP2 protein (Fragment)
PSMC3	1.32E+07	70.2	49.2	26S proteasome regulatory subunit 6A
FAM49B	1.32E+07	21.6	36.7	Family with sequence similarity 49, member B
PSMD13	1.22E+07	30.4	42.6	26S proteasome non-ATPase regulatory subunit 13 (Fragment)
CTSA	1.12E+07	25.8	54.2	cDNA FLJ60397, highly similar to Lysosomal protective protein
APOH	1.10E+07	16.3	30.3	Apolipoprotein H
C2orf69	1.08E+07	6.3	13.3	Chromosome 2 Open Reading Frame 69
CAPG	1.06E+07	45.8	38.5	Macrophage-capping protein
PON2	1.06E+07	19.4	40	Paraoxonase
LRPAP1	1.05E+07	34.9	41.5	LDL Receptor Related Protein Associated Protein 1
LGALS3	1.05E+07	8.0	27.1	Galectin (Fragment)
PIP	1.03E+07	14.0	16.6	Prolactin-inducible protein
LGALS7	9.90E+06	26.6	15.1	Galectin-7
ARSA	9.70E+06	31.3	53.8	Arylsulfatase A
LPL	9.65E+06	31.2	51.7	Lipoprotein lipase
LOX	9.50E+06	23.3	46.9	Lysyl oxidase homolog
RGN	9.22E+06	13.1	33.2	Regucalcin
SERPINB3	8.88E+06	9.8	44.5	Serpin B3
CFD	8.34E+06	17.8	24.4	Adipsin
GYG1	8.31E+06	18.4	21.5	Glycogenin 1
PSMA2	8.30E+06	47.5	25.9	Proteasome subunit alpha type-2
HMGB2	8.19E+06	16.7	24	High mobility group protein B2
APOA1BP; NAXE	8.16E+06	15.2	33.6	NAD(P)H-hydrate epimerase
CAPZA2	8.09E+06	26.0	32.9	F-actin-capping protein subunit alpha-2
ARPC5	7.55E+06	11.3	16.3	Actin-related protein 2/3 complex subunit 5
S100A10	7.00E+06	24.0	11.2	S100 Calcium Binding Protein A10
SRP14	6.09E+06	15.6	14.6	Signal recognition particle 14 kDa protein
NOV	5.87E+06	16.6	39.1	CCN family member 3
MAPK1	5.67E+06	22.2	41.4	Mitogen-activated protein kinase
HTRA1	5.53E+06	7.8	48.4	HtrA Serine Peptidase 1
EDIL3	5.40E+06	13.1	53.7	EGF-like repeat and discoidin I-like domain-containing protein 3
CAB39	5.20E+06	6.6	39.7	MO25-like protein

AZGP1	5.19E+06	15.2	34.2	Testicular tissue protein Li 227
NEGR1	5.03E+06	7.6	38.7	Neuronal growth regulator 1
PSMD7	4.30E+06	21.6	28.3	Proteasome 26S Subunit, Non-ATPase 7
CPA4	4.28E+06	5.8	47.3	Carboxypeptidase A4
PROCR	4.07E+06	8.8	26.7	Endothelial protein C receptor
SDCBP	3.48E+06	5.4	32.4	Syndecan binding protein (Syntenin)
CHID1	3.39E+06	7.7	48.2	Chitinase Domain Containing 1
S100A11	3.36E+06	8.2	11.7	S100 Calcium Binding Protein A11
PSMD14	3.22E+06	30.2	34.6	26S proteasome non-ATPase regulatory subunit 14
S100A13	2.83E+06	12.1	11.5	S100 Calcium Binding Protein A13
SERPINA7	2.59E+06	5.2	46.3	Thyroxine-binding globulin
TFPI	2.46E+06	11.6	35	Tissue factor pathway inhibitor
VTN	2.26E+06	6.9	54.3	Vitronectin
OGN	2.25E+06	14.6	30.4	Mimecan (Fragment)
TWSG1	2.13E+06	7.4	25	Twisted gastrulation protein homolog 1
DBNL	2.03E+06	22.0	48.2	Drebrin-like protein
ULBP2	2.02E+06	5.4	27.4	UL16-binding protein 2
CANT1	1.94E+06	18.8	44.8	Calcium activated nucleotidase 1
TGFB2	1.62E+06	10.1	47.7	Transforming growth factor beta
MAPK14	1.58E+06	15.9	41.3	Mitogen-activated protein kinase 14
CSTB	1.51E+06	25.9	11.1	Cathepsin B
DERA	1.40E+06	10.8	35.2	Deoxyribose-phosphate aldolase
IGFBP6	1.30E+06	7.5	25.3	Insulin-like growth factor-binding protein 6
SRGN	1.27E+06	5.1	17.6	Proteoglycan 1, secretory granule
NEU1	1.23E+06	6.5	45.4	Exo-alpha-sialidase
RNASE7	1.18E+06	6.0	17.4	Ribonuclease 7
PAFAH1B2	1.16E+06	8.7	25.6	Epididymis secretory protein Li 303
ALAD	9.53E+05	17.6	37.2	Delta-aminolaevulinic acid dehydratase
RBP4	9.20E+05	17.3	23	Retinol-binding protein
BGN	8.67E+05	11.9	37.6	Biglycan
ECM1	8.63E+05	6.6	45.5	Truncated extracellular matrix protein 1
SHBG	7.93E+05	6.4	26.1	Sex hormone-binding globulin
THEM6	7.43E+05	7.1	23.9	Protein THEM6
NTNG1	5.31E+05	7.6	40.5	Netrin G1

Table V-4. Ranked abundance of proteins detected in the <50 kDa fraction of DENV-infected HeLa supernatant in comparison to uninfected HeLa cell culture supernatant (conditioned media) (n=1). Differences expressed as Log2-fold change. Proteins highlighted in blue are members of the complement family and proteins highlighted in yellow are implicated in DENV replication or pathogenesis.

	Gene Name	<50 kDa		Conditioned Media		Molecular Weight (kDa)	Protein Identified	Abundance in <50 kDa fraction	Log2-Fold change (<50 kDa:Conditioned Media)
		Ranked Abundance (Percentile)	Peptide score	Ranked Abundance (Percentile)	Peptide score				
Unique to <50 kDa	**CALM3**	38 (24%)	106.0	**ND**	**ND**	16.8	Calmodulin-3	1.0E+08	ND
	ACTR2	63 (39%)	77.2	**ND**	**ND**	44.7	Actin-related protein 2	4.1E+07	ND
	PSMC2	66 (41%)	58.9	**ND**	**ND**	48.6	26S proteasome AAA-ATPase subunit RPT1	3.7E+07	ND
	PSMD12	71 (44%)	10.7	**ND**	**ND**	52.9	26S proteasome non-ATPase regulatory subunit 12	3.3E+07	ND
	HEBP2	94 (59%)	6.8	**ND**	**ND**	24	HEBP2 protein (Fragment)	1.4E+07	ND
	PSMC3	95 (59%)	70.2	**ND**	**ND**	49.2	26S proteasome regulatory subunit 6A	1.3E+07	ND
	CTSA	98 (61%)	25.8	**ND**	**ND**	54.2	Cathepsin A	1.1E+07	ND
	C2orf69	100 (63%)	6.3	**ND**	**ND**	13.3	Chromosome 2 Open Reading Frame 69	1.1E+07	ND
	PON2	102 (64%)	19.4	**ND**	**ND**	40	Paraoxonase	1.1E+07	ND
	LGALS3	104 (65%)	8.0	**ND**	**ND**	27.1	Galectin (Fragment)	1.0E+07	ND
	LGALS7	106 (66%)	26.6	**ND**	**ND**	15.1	Galectin-7	9.9E+06	ND
	SERPINB3	111 (69%)	9.8	**ND**	**ND**	44.5	Serpin B3	8.9E+06	ND
	PSMA2	114 (71%)	47.5	**ND**	**ND**	25.9	Proteasome subunit alpha type-2	8.3E+06	ND
	NAXE	116 (73%)	15.2	**ND**	**ND**	33.6	NAD(P)H-hydrate epimerase	8.2E+06	ND
	CAPZA2	117 (73%)	26.0	**ND**	**ND**	32.9	F-actin-capping protein subunit alpha-2	8.1E+06	ND
	ARPC5	118 (74%)	11.3	**ND**	**ND**	16.3	Actin-related protein 2/3 complex subunit 5	7.5E+06	ND

	SRP14	120 (75%)	15.6	**ND**	**ND**	14.6	Signal recognition particle 14 kDa protein	6.1E+06	ND
	HTRA1	123 (77%)	7.8	**ND**	**ND**	48.4	HtrA Serine Peptidase 1	5.5E+06	ND
	AZGP1	126 (79%)	15.2	**ND**	**ND**	34.2	Testicular tissue protein Li 227	5.2E+06	ND
	PSMD7	128 (80%)	21.6	**ND**	**ND**	28.3	Proteasome 26S Subunit, Non-ATPase 7	4.3E+06	ND
	CHID1	132 (83%)	7.7	**ND**	**ND**	48.2	Chitinase Domain Containing 1	3.4E+06	ND
	PSMD14	134 (84%)	30.2	**ND**	**ND**	34.6	26S proteasome non-ATPase regulatory subunit 14	3.2E+06	ND
	TFPI	138 (86%)	11.6	**ND**	**ND**	35	Tissue factor pathway inhibitor	2.5E+06	ND
	VTN	139 (87%)	6.9	**ND**	**ND**	54.3	Vitronectin	2.3E+06	ND
	TWSG1	141 (88%)	7.4	**ND**	**ND**	25	Twisted gastrulation protein homolog 1	2.1E+06	ND
	DBNL	142 (89%)	22.0	**ND**	**ND**	48.2	Drebrin-like protein	2.0E+06	ND
	ULBP2	143 (89%)	5.4	**ND**	**ND**	27.4	UL16-binding protein 2	2.0E+06	ND
	CANT1	144 (90%)	18.8	**ND**	**ND**	44.8	Calcium activated nucleotidase 1	1.9E+06	ND
	TGFB2	145 (91%)	10.1	**ND**	**ND**	47.7	Transforming growth factor beta	1.6E+06	ND
	MAPK14	146 (91%)	15.9	**ND**	**ND**	41.3	Mitogen-activated protein kinase 14	1.6E+06	ND
	DERA	148 (93%)	10.8	**ND**	**ND**	35.2	Deoxyribose-phosphate aldolase	1.4E+06	ND
	IGFBP6	149 (93%)	7.5	**ND**	**ND**	25.3	Insulin-like growth factor-binding protein 6	1.3E+06	ND
	SRGN	150 (94%)	5.1	**ND**	**ND**	17.6	Proteoglycan 1, secretory granule	1.3E+06	ND
	NEU1	151 (94%)	6.5	**ND**	**ND**	45.4	Neuraminidase 1	1.2E+06	ND
	RNASE7	152 (95%)	6.0	**ND**	**ND**	17.4	Ribonuclease 7	1.2E+06	ND
	PAFAH1B2	153 (96%)	8.7	**ND**	**ND**	25.6	Epididymis secretory protein Li 303	1.2E+06	ND
	ALAD	154 (96%)	17.6	**ND**	**ND**	37.2	Delta-aminolaevulinic acid dehydratase	9.5E+05	ND
	BGN	156 (98%)	11.9	**ND**	**ND**	37.6	Biglycan	8.7E+05	ND
	ECM1	157 (98%)	6.6	**ND**	**ND**	45.5	Truncated extracellular matrix protein 1	8.6E+05	ND

	NTNG1	160 (100%)	7.6	**ND**	**ND**	40.5	Netrin G1	5.3E+05	ND
>2-fold Change	**TUBB**	13 (8%)	194.5	124 (97%)	64.3	47.7	Tubulin beta chain (Fragment)	3.9E+08	7.67
	PSMD6	35 (22%)	48.5	120 (94%)	8.0	45.5	26S proteasome non-ATPase regulatory subunit 6	1.1E+08	5.58
	PSMD11	73 (46%)	48.2	125 (98%)	10.0	47.4	26S proteasome non-ATPase regulatory subunit 11	3.1E+07	4.07
	S100A13	136 (85%)	12.1	128 (100%)	7.6	11.5	S100 Calcium Binding Protein A13	2.8E+06	3.78
	TAGLN2	69 (43%)	81.5	114 (89%)	26.3	22.4	Transgelin-2	3.4E+07	3.24
	PPIE	85 (53%)	33.1	122 (95%)	9.4	24.1	Peptidyl-prolyl cis-trans isomerase	1.9E+07	3.19
	HNRNPC	30 (19%)	62.9	72 (56%)	22.2	32.0	Heterogeneous nuclear ribonucleoproteins C1/C2	1.5E+08	2.82
	HMGB2	115 (72%)	16.7	127 (99%)	5.6	24.0	High mobility group protein B2	8.2E+06	2.69
	FAM49B	96 (60%)	21.6	121 (95%)	11.4	36.7	Family with sequence similarity 49, member B	1.3E+07	2.53
	NIT2	79 (49%)	47.0	112 (88%)	19.3	30.6	Omega-amidase NIT2	2.5E+07	2.47
	ARG1	33 (21%)	37.6	69 (54%)	46.2	34.7	Arginase-1	1.2E+08	2.31
	APRT	70 (44%)	28.0	95 (74%)	8.9	19.6	Adenine phosphoribosyltransferase	3.4E+07	2.06
	PSMB1	46 (29%)	43.9	80 (63%)	16.4	26.5	Proteasome subunit beta type-1	7.4E+07	2.01
	PSMA5	56 (35%)	43.5	83 (65%)	22.1	26.4	Proteasome subunit alpha type	5.3E+07	1.73
	LPL	108 (68%)	31.2	116 (91%)	14.4	51.7	Lipoprotein lipase	9.7E+06	1.71
	AIMP1	88 (55%)	46.2	105 (82%)	22.2	34.3	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1	1.8E+07	1.61
	ILF2	57 (36%)	84.0	82 (64%)	29.2	38.9	Interleukin enhancer-binding factor 2	5.2E+07	1.60
	TUBA4A	6 (4%)	281.2	21 (16%)	131.2	49.9	Tubulin alpha-4A chain	7.4E+08	1.56
	CAP1	58 (36%)	97.7	78 (61%)	29.8	51.6	Adenylyl cyclase-associated protein 1	5.1E+07	1.45
	CYB5R3	78 (49%)	44.9	93 (73%)	23.9	33.2	NADH-cytochrome b5 reductase	2.5E+07	1.41
	FUCA1	91 (57%)	22.7	107 (84%)	11.6	53.7	Tissue alpha-L-fucosidase	1.5E+07	1.41
	PRDX4	49 (31%)	97.1	71 (55%)	33.4	30.5	Peroxiredoxin-4	6.4E+07	1.40
	SERPINB5	48 (30%)	63.0	63 (49%)	27.9	42.1	Serpin B5	7.3E+07	1.33

	S100A4	39 (24%)	17.2	55 (43%)	11.6	11.7	S100 Calcium Binding Protein A4	1.0E+08	1.28
	CALR	84 (53%)	54.1	94 (73%)	30.9	46.9	Calreticulin variant (Fragment)	2.0E+07	1.20
	PRDX6	24 (15%)	131.0	34 (27%)	55.4	25.0	Peroxiredoxin-6	1.8E+08	1.14
	HNRNPA2B1	9 (6%)	187.2	20 (16%)	109.7	37.4	Heterogeneous nuclear ribonucleoproteins A2/B1	5.7E+08	1.11
	CALU	29 (18%)	144.3	37 (29%)	67.1	37.1	Calumenin	1.6E+08	1.10
	EFEMP1	74 (46%)	37.4	85 (66%)	15.4	45.7	EGF containing fibulin-like extracellular matrix protein 1 isoform 2 (Fragment)	3.1E+07	1.06
	EEF1A1	5 (3%)	177.9	11 (9%)	75.6	50.2	Elongation factor 1-alpha (Fragment)	7.7E+08	1.00
<2-fold Change	**LOX**	109 (68%)	23.3	76 (59%)	22.3	46.9	Lysyl oxidase homolog	9.5E+06	-1.02
	HBA2	41 (26%)	31.4	24 (19%)	30.6	15.2	Haemoglobin subunit alpha	8.5E+07	-1.04
	CFD	112 (70%)	17.8	79 (62%)	14.9	24.4	Adipsin /Complement Factor D	8.3E+06	-1.15
	CST3	31 (19%)	38.0	12 (9%)	36.3	15.8	Epididymis secretory protein Li 2	1.3E+08	-1.41
	THEM6	159 (99%)	7.1	123 (96%)	5.3	23.9	Protein THEM6	7.4E+05	-1.43
	OGN	140 (88%)	14.6	103 (80%)	23.8	30.4	Mimecan (Fragment)	2.2E+06	-1.47
	EDIL3	124 (78%)	13.1	84 (66%)	15.0	53.7	EGF-like repeat and discoidin I-like domain-containing protein 3	5.4E+06	-1.51
	CPA4	129 (81%)	5.8	87 (68%)	11.7	47.3	Carboxypeptidase A4	4.3E+06	-1.66
	SHBG	158 (99%)	6.4	118 (92%)	6.6	26.1	Sex hormone-binding globulin	7.9E+05	-1.70
	AFP	15 (9%)	35.5	4 (3%)	25.6	50.7	Alpha Fetoprotein	3.7E+08	-1.75
	H3F3A	44 (28%)	9.0	14 (11%)	11.3	15.3	Histone H3.3	8.3E+06	-1.93
	SERPINA7	137 (86%)	5.2	90 (70%)	7.2	46.3	Thyroxine-binding globulin	2.6E+06	-2.02
	SERPINF1	50 (31%)	31.7	15 (12%)	38.8	46.3	Pigment epithelium-derived factor	6.2E+07	-2.25
	RGN	110 (69%)	13.1	45 (35%)	23.6	33.2	Regucalcin	9.2E+06	-2.54
	CSTB	147 (92%)	25.9	89 (70%)	12.9	11.1	Cathepsin B	6.1E+07	-2.83
	RBP4	155 (97%)	17.3	57 (45%)	19.2	23.0	Retinol-binding protein	9.2E+05	-5.24

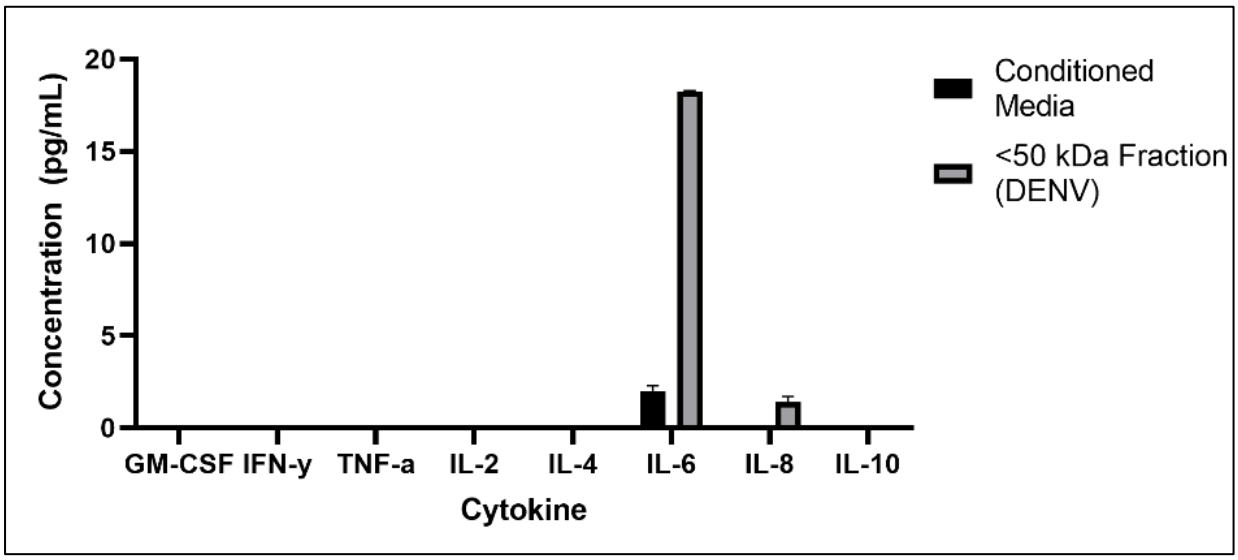

Figure V-3. HeLa produce increased IL-6 and IL-8 in response to DENV infection.

Supernatant of uninfected HeLa and the DENV infected (MOI=1) HeLa were collected 48 hpi and DENV-infected supernatant filtered for small (<50 kDa) secreted proteins and analysed by Bio-Plex cytokine array. DENV = Dengue Virus, GM-CSF = Granulocyte-macrophage colony-stimulating factor, IFN = interferon, TNF-α = tumour necrosis factor-α, IL=interleukin. Results are representative of n=1 for 2 technical replicates, error bars represent the mean and standard deviation for assay replicates. Statistical analysis not performed.

V.3 Discussion

The experiments in this chapter used both hypothesis-driven, targeted techniques (RT-qPCR and IF) as well as unbiased, descriptive proteomic research methods (mass spectrometry) to define the agents that induce FH mRNA and FH cell association in DENV infected cells. These data narrow the pool of candidates for FH mRNA induction, further defined complement production in response to DENV infection and produced a freely accessible mass spectrometry dataset for DENV-infected HeLa. The utility of public access to large datasets has already been emphasised by the results produced in **Chapter III**.

Size-fractionation of supernatants from DENV-infected cells into >50 and <50 kDa proteins was performed in an attempt to separate larger proteins from smaller cytokines and chemokines, as likely candidates for stimulating FH.[161, 181, 360] Surprisingly, acute FH mRNA induction only occurred in >50 kDa-treated cells but not by other treatments and FH mRNA induction was not attributable to the amount of DENV RNA at 8 hpi. LPS has been shown previously to increase FH mRNA in MDM[44] which is likely to occur through TLR-4-mediated NF-κB activation whereas Poly I:C induces FH mRNA, likely mediated by activation of intracellular PRRs (described in **Section I-1.9.1**). Additionally, secreted DENV-NS1, as found in the >50 kDa supernatant fraction, has been shown to activate TLR-4[64] and may therefore contribute to FH mRNA upregulation induced by the >50 kDa supernatant fraction. Alternatively, virions may trigger the same innate immune pathways activated by Poly I:C, and investigating the DENV proteins secreted during infection may therefore be an important target for future investigation. The effect of DENV but not DENV replication on FH mRNA induction could be assessed by stimulating cells with either infectious and heat inactivated DENV and quantitating FH mRNA by qPCR as above. Induction of FH mRNA by the large >50 kDa fraction of supernatant but not unfiltered supernatant, may be suggestive of an inhibitor of FH mRNA induction in the <50 kDa fraction. The inhibitory effects of the agents in the <50 kDa supernatant fraction could be evaluated by performing DENV infection in cells pre-treated, concomitantly treated and treated post infection with the <50 kDa supernatant fraction. This was the method previously performed by Diamond *et al.,* (2000)[93] when evaluating the stimulation of antiviral effects by IFNs during DENV infection. FB mRNA induction did not follow the same pattern of FH mRNA induction, as FB mRNA increased for cells treated with unfiltered supernatant. Potentially, agents that induce FB mRNA in the unfiltered supernatant do not induce FH mRNA.

Results of the IF microscopy demonstrated that acute FH mRNA induction, observed by qPCR, was not required to increase FH-cell association. DENV (MOI=1) infected cells had the highest proportion of cells actively replicating DENV, despite approximately 10-fold less DENV RNA than unfiltered supernatant by 8 hpi. This demonstrates that DENV RNA at 8 hpi did not correspond with DENV replication by 24 hpi for the supernatant fractions. DENV infected cells also had the most cell-associated FH by 24 hpi, despite no evidence of acute FH mRNA induction. This may be due to mechanisms that promote FH recruitment to the cell, induced by small molecules. Conversely unfiltered supernatant-treated cells displayed low numbers of dsRNA positive cells, despite high DENV RNA at 8 hpi. Plaque assay to accompany these PCR data would provide insight into the amount of infectious virus detectable by 8 hours. However, as the aim of this experiment was to assess the acute response of cells to secreted agents, the amount of infectious virus observed by IF is not meaningfully informed further by the addition of a plaque assay. Increased cell associated FH in cells treated with <50 kDa supernatant fraction demonstrated FH recruitment was independent of DENV virions, large proteins, and FH mRNA induction. Paired RT-qPCR data from cells harvested 24 hpi, as performed in preparation for IF microscopy, would assist interpretation of the results for IF microscopy further. These qPCR data could be used to determine whether FH mRNA induction was detectable by 24 hpi, when FH-cell association is observable. However, in MDM and EC, FH mRNA induction following DENV-infection is not detectable by 24 hpi.[44]

In consideration of the qPCR and IF results together, DENV RNA from DENV-infected HeLa supernatant did not correspond with infectivity of the supernatant. This may be a factor of supernatant preparation, potentially by the shear stress introduced to virions during centrifugation, inactivating them. Alternatively, reduced infectivity may be explained by the presence of a large protein that induces an antiviral state in naïve cells, which would be an interesting concept for future research. Furthermore, FH mRNA induction did not directly result in increased cell-associated FH as observed by IF microscopy. Instead, cells that did not have FH mRNA induced by 8 hpi (DENV and <50 kDa-treated) appeared to have some of the strongest cell-associated FH by 24 hpi. This may be due to changes in FH protein recruitment, e.g. due to changes in extracellular GAGs as described in **Chapter IV** or FH internalisation by early apoptotic cells, described in pp. 104. Induction of FH mRNA by the >50 kDa fraction and induction of cell-association by the <50 kDa fraction did not occur when these components were combined i.e. in the unfiltered supernatant. However, when

separated, the >50 kDa secreted proteins acutely induce FH mRNA and a minor increase in FH-cell association. Conversely, <50 kDa supernatant fraction did not significantly increase FH mRNA but contained stimuli that appeared capable of increasing FH-cell association. Use of software and technology capable of quantitating immunofluorescent signal, such as Operetta (PerkinElmer) could improve the utility of these IF microscopy experiment further. After observing distinctions between FH mRNA induction and protein localisation in response to different molecular weight stimuli, the fractions of the DENV-infected HeLa supernatant were investigated by mass spectrometry.

The FH protein was detectable within the unfiltered and >50 kDa supernatant fraction of DENV-infected cells by mass spectrometry but was not amongst the top 100 secreted and extracellular proteins. Mass spectrometry also identified that the unfiltered and >50 kDa supernatant fraction contained the three DENV structural proteins (capsid, envelope, and membrane), most likely present as assembled virions and hence retained in the >50 kDa fraction. The >50 kDa fraction also contained DENV-NS1, probably as the 310 kDa secreted hexamer and not as the 52 kDa monomer that only exists briefly following translation, nor as the intracellular and membrane-bound dimer.[60] Secreted DENV-NS1 is a potential candidate for FH mRNA induction due to reports that it can activate TLR-4.[44, 64, 361] Increased FB and C5 following DENV infection is well supported by patient (C5, FB) and *in vitro* (FB) data.[43, 44, 362] FB and C5 may induce FH mRNA through C5a generation, via C5a receptor (C5aR) activation[363], inducing NF-κB, although this remains to be investigated further. To address this, cells could be treated with C5a and FH mRNA quantitated by qPCR or performing C5 depletion using antibodies on the >50 kDa supernatant fraction or by the $C5aR_1$ antagonist avacopan[154].

Although comparative abundance of proteins detected by mass spectrometry analysis was not quantitative, some proteins shifted from low-confidence, low-abundance in conditioned media to the highest abundance in the >50 kDa supernatant fraction of DENV-infected cells. These proteins would be interesting targets for future research, and included C4, α_2M and ITIH2. C4 is the subunit of the C3-convertase used by both the CP and MBL[364] and is therefore integral to the complement cascade but there is no indication that it induces FH mRNA production, except perhaps by facilitating C5a generation. The protease inhibitor and chaperonin α_2M is a member of the complement-coagulation family, improves DENV virion stability and worsens WNV and JEV neuropathology in mice.[365, 366] α_2M is also acutely increased in patients that develop dengue with complications compared to dengue

controls.[367] Increased ITIH2 following DENV infection has not been recorded, although there is evidence to suggest *in vitro* that ITIH2 is necessary for DENV to enter and infect dendritic cells.[368] ITIH2 has no known complement interactions and α_2M has some interaction with complement. Importantly, both of these proteins are known to interact with DENV. Thus, the induction of these proteins reinforces that HeLa are a valuable *in vitro* model for investigating host-cell responses to DENV infection.

The proteins uniquely detected in the <50 kDa supernatant fraction by mass spectrometry did not include any cytokines or chemokines, which were originally thought to be driving FH mRNA induction during DENV-infection. Mass spectrometry is an unbiased quantitative analysis of small peptides produced by sample digestion, utilising known-protein compositions to determine the abundance of thousands of unique proteins in a given sample. However, low molecular weight proteins such as cytokines can effect cells at very low concentrations and typically suffer from short half-lives. Additionally, cytokine detection is complicated by the presence of larger proteins found in high concentration such as albumin and immunoglobulin, causing challenges for cytokine quantitation by mass spectrometry.[369] Bio-plex arrays, however, perform antibody-based detection of up to 48 proteins per assay and have high sensitivity that is independent of other proteins in a sample.[370] A pilot Bio-plex cytokine array analysis revealed that IL-6 and IL-8 were increased in the unfiltered and <50 kDa fraction of the supernatant following DENV infection. The literature supports DENV-mediated IL-6 and IL-8 elevation which is reported in dengue patients[371-373] and *in vitro*[374]. Importantly, higher concentrations of IL-6 in patient blood correlate with greater disease severity.[16] FH mRNA is not induced by IL-6 *in vitro* for U118-MG astroglioma or Hep2b hepatoma cells[354] which aligns with the observation that the <50 kDa supernatant fraction did not induce FH mRNA. Further analysis of the supernatants by a wider array of cytokine detection was cost prohibitive but demonstrates that mass spectrometry was not able to replace antibody-based detection methods for cytokines and chemokines in cell culture supernatant.

Reduction of the serine protease, FD, within the <50 kDa fraction of DENV-infected HeLa supernatant was observed and is in alignment with observations made in dengue patients.[217] FD within the <50 kDa fraction of HeLa supernatant was approximately 2-fold lower than in conditioned media. Dengue patients but not severe dengue patients have decreased circulating FD during the acute stage of disease.[217] As FD is the rate-limiting factor for AP activity, this is thought to protect the patient from excessive AP activation and ensuing

complementopathy.[139] Increased α_2M was detected in the >50 kDa fraction and will inactivate and direct destruction of any protease. It is therefore tempting to speculate that a relationship might exist between elevated α_2M and decreased serine protease FD *in vitro*. Activated α_2M is a cage-like protein that traps proteases which cleave the α_2M internal bait region.[365] Therefore, demonstrating FD-α_2M binding would not convey the same importance as demonstrating sequestration and trapping. FD-α_2M interactions have not yet been published. However, this interaction could be investigated by pulsing activated or inactive α_2M with FD and detergent treating samples to dissociate bound but not trapped proteins. Then, α_2M immunoprecipitation could be performed and the unprecipitated (not trapped) FD quantitated by ELISA or Western blot.

Vitronectin was detected in the <50 kDa fraction of the supernatant but not conditioned media. Vitronectin is a small complement regulator that inhibits MAC formation, preventing complement mediated cell death.[375] Secreted DENV-NS1 will bind vitronectin *in vitro* to protect infected cells from complement, however this is the first evidence that DENV may also be elevating vitronectin abundance.[234] It is worth noting that increasing inhibition of complement at the phase of MAC formation, as performed by vitronectin, is likely deleterious to the host. Vitronectin will rescue the cell, but only after C3- and C5-convertase formation, exponentially increasing the abundance of active C3b and C5b, accompanied by increased circulating anaphylatoxins C3a and C5a. This emphasises the importance of FH, where FH-mediated AP regulation prevents and disrupts convertase formation and therefore regulates complement activity before AP amplification. Investigating the actions of vitronectin during DENV-infection would be worthwhile, where MAC formation and cell death could be quantitated during DENV infection in the presence and absence of vitronectin using CRISPR vitronectin$^{-/-}$ knockout cell line models.

V.3.1 Limitations

Due to pandemic-related restrictions on both facility access and reagents, pilot mass spectrometry and cytokine array experiments performed in this chapter were only able to be performed once and need repeating. Additionally, due to limited access to resources, conditioned media controls were not also fractionated by size. Therefore, unique detection of some proteins in the >50 kDa and <50 kDa fractionated supernatants might occur on a smaller pool of total proteins. However, most trends observed for the proteins within the fractionated supernatant were seen in the unfiltered supernatant also. Examples of unique proteins, such as FB, that were marginally above the lower limit of detection, have instead

been corroborated by evidence in the literature and observations herein **(Section III.2.1-4 and V.2.1)**.

V.4 Summary

DENV infection causes secretion of large proteins capable of inducing FH mRNA *in trans* in DENV naïve HeLa. The small, secreted, proteins require further investigation with targeted analysis of the effect produced by individual cytokines, especially focussing on whether they have the ability to recruit FH to cells. Some 49 proteins were uniquely detected in the supernatant of DENV-infected HeLa, highlighting the changes DENV elicits in the secretome of infected cells. The proteins released following DENV infection included complement AP activators and late-stage regulators that favour complement AP amplification over control. These proteins may represent factors that contribute to dengue pathogenesis and potential therapeutic targets for future investigation.

CHAPTER VI

DISEASE PROFILES IN THE INDIGENOUS AUSTRALIAN POPULATION ARE SUGGESTIVE OF A COMMON COMPLEMENT GENOTYPE

VI.1 Introduction

In conversations with the researchers in the Flinders Ophthalmology team, it was commented that Indigenous Australians, who are disparately affected by many diseases, rarely develop age related macular degeneration (AMD) (Professor Jamie Craig and Mr Jose Estevez, personal communications (2020)). The link between AMD and polymorphisms in CFH and other AP proteins have been defined extensively.[171, 172, 376, 377] Thus, in consideration of complotype interaction with AMD and other diseases, a review of the literature was performed, and a hypothesis was generated. The following chapter is presented as an adapted form of the manuscript under review for publication at time of submission.

The complement system is ancient, with the first complement analogues evolving 700 million years ago in sea urchins, some human complement regulatory genes have only been acquired in higher-order primates as recently as 19 million years ago.[253, 378] These more evolutionarily recent complement components, such as *CFHR* genes, have heterogenous haplotypes worldwide that cluster by ethnicity, resulting in populations with distinct complement phenotypes.[203, 379] For example, European populations have a different haplotype of complement regulatory components when compared to Sub-Saharan African populations, which is proposed to be driven by selective pressures of autochthonous infectious diseases.[253, 378] The combination of conserved and evolutionarily newer and more variable complement proteins forms a 'complotype': the combination of genetic variants within complement genes.

International efforts to characterise complotypes have revealed copy number variants of the *CFHR* family commonly grouped by genetic ancestry.[203] As each complotype interacts with infectious and autoimmune diseases differently, commonalities in diseases attributed to responses to infection and complementopathy can suggest similar complotypes. The Aboriginal and Torres Strait Islander population of Australia (respectfully referred to as Indigenous Australians hereafter) are the oldest living culture globally and suffer from substantial health inequity today as a consequence of colonisation, dispossession, and political oppression, including high rates of infectious diseases.[380] For reasons discussed herein, we hypothesise that Indigenous Australians harbour a relatively common genetic variation in the recently acquired

concomitant deletion of *CFHR1* and *CFHR3* (*CFHR3-1Δ*), effecting their complotype. In this review, data on adverse autoimmune disease occurrence in Indigenous Australians and, altered susceptibility to infections known to be associated with the *CFHR3-1Δ* haplotype, have been critically assessed. This, provides evidence to support future studies of this common complement haplotype in Indigenous Australians, which may ultimately impact on health outcomes in this vulnerable population.

VI.2 Hypothesis

Hypothesis: The pattern of specific diseases in people with Indigenous Australian ancestry is indicative of the presence of the *CFHR3-1Δ* complotype.

VI.3 *CFHR3-1Δ*: A common CFHR haplotype in humans that influences autoimmune and infectious diseases

CFHR3 and *CFHR1* are situated in tandem on chromosome 1, downstream of *CFH*. (30) Flanking the 5' end of *CFHR3* and the 3' end of *CFHR1* are 29 kb duplicated regions of the chromosome. The genotype *CFHR3-1Δ* is an 86.4 kb deletion caused by excision of these genes by nonallelic homologous recombination at the 29kb regions flanking both genes **(Figure VI-1)**.[258, 381] Interestingly, *CFHR3-1Δ* is protective of some diseases at the expense of an increased risk of others. For instance, *CFHR3-1Δ* protects from the development of AMD and IgA nephropathy (IgAN).[203, 382, 383] Conversely, *CFHR3-1Δ* increases the risk of developing rarer autoimmune disorders such as systemic lupus erythematosus (SLE) and FH autoantibodies causing atypical haemolytic uraemic syndrome.[197, 203, 220, 279, 381-384] Due to the phenomena of the founder effect coupled with selective pressures, the prevalence of the *CFHR3-1Δ* haplotype in a population is geographically associated with endemic infectious diseases that interact with the FHR proteins.[203] The *CFHR3-1Δ* haplotype decreases the risk of diseases such as malaria and leprosy, which have been proposed to drive selection of the haplotype in a population, as in Sub-Saharan Africans, who have the highest rates of *CFHR3-1Δ* in the world.[385-388]

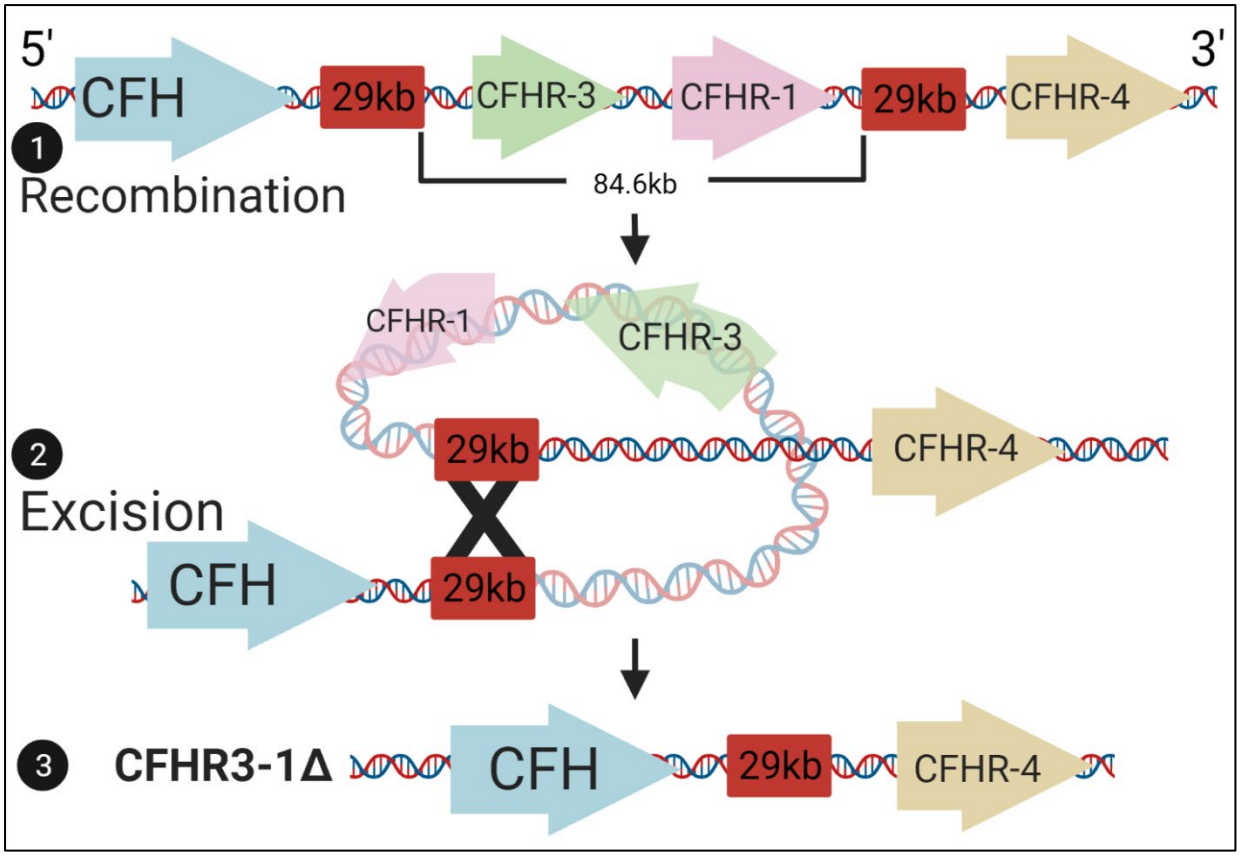

Figure VI-1. Recombination and excision of *CFHR1* and *CFHR3* results in the *CFHR3-1Δ* haplotype

CFH, CFHR-3, -1 and -4, with CFHR-3 and -1 on chromosome 1q32 are flanked by identical 29 kb sequences. These 29 kb repeat sequences can undergo (1) recombination, followed by (2) excision of *CFHR1* and *CFHR3* genes (84.6 kb), leaving (3) the *CFHR3-1Δ* haplotype, lacking *CFHR1* and *CFHR3* genes. FHR = factor H related.

VI.4 Evidence for higher frequencies of *CFHR3-1Δ* among Indigenous Australians based on non-infectious disease prevalence: low rates of IgAN and AMD but high rates of SLE.

Due to numerous socioeconomic, political, and environmental pressures, Indigenous Australians are disproportionately burdened by various infectious and chronic diseases compared to non-Indigenous Australians with European ancestry.[380, 389] Indigenous Australians are known to have lower rates of IgAN and AMD and higher rates of SLE: a disease pattern that is typical of the *CFHR3-1Δ* haplotype. Alternatively, 70,000 years of separation may have driven the evolution of a novel complotype that mimics the disease prevalence in populations with *CFHR3-1Δ*, although, this seems less likely.

Chronic kidney disease is a major cause of morbidity and mortality for Indigenous Australians, with end-stage kidney failure (ESKF) prevalence approximately 6-fold higher and occurring 30 years younger in the Indigenous Australian population.[390, 391] However, analysis of renal biopsies suggests that IgAN is less prevalent among Indigenous Australians with ESKF (between 12.7-19.1% Indigenous Australians versus 24.4% for non-Indigenous Australians, p = 0.001), despite a predisposition to other kidney diseases **(Table VI-1)**.[392]

Similarly, while AMD is a leading cause of blindness and vision impairment among non-Indigenous Australians, contributing to 10.3% of all vision loss cases, AMD among Indigenous Australian people is uncommon. Since increasing age is a significant risk factor for developing AMD, the shorter life expectancy of Indigenous Australians may contribute, at least in part, to these observations.[393] In the National Indigenous Eye Health Survey, vision loss because of advanced AMD was infrequently reported (prevalence of 0.95% vs 10.3% Indigenous Australian vs non-Indigenous Australian), and in the Central Australia Ocular Health Study, there was not a single case of vision loss for any of the study participants from AMD (study size >1300).[394] More recently, the Australian National Eye Health Survey found rates among non-Indigenous Australians for intermediate and late AMD to be 10.5% and 0.96%, respectively. By comparison, the prevalence of intermediate AMD was 5.7%, and for late AMD, only three cases (0.17%) were found among Indigenous Australians compared to 33 cases among non-Indigenous Australians **(Table VI-1)**.[221] Hence, AMD-related vision loss and clinical findings consistent with AMD are less common amongst Indigenous

Australians, consistent with a population with higher frequencies of the *CFHR3-1Δ* haplotype.

Although protection from IgAN and AMD are known benefits of the *CFHR3-1Δ* haplotype, the *CFHR3-1Δ* haplotype increases the risk of SLE.[258, 382] Consistent with this, Indigenous Australians are disproportionately affected by SLE.[210, 350, 395-400] In 2001, the prevalence of SLE in Indigenous Australians living in Central Australia (1 per 1360) was approximately 4-fold higher than in Caucasians from the same geographical area (1 per 5170) **(Table VI-1)**.[396] The disparity of SLE prevalence observed in Indigenous Central Australian communities was also observed in other Indigenous communities from Northern Territory.[397] In a study of 24,900 people, Indigenous Australians had 1 case of SLE per 1900 people, approximately twice the prevalence of SLE when compared to the reported national average of 1 per 4,000.[397] Additionally, two separate studies conducted in far North Queensland communities described rates of SLE in two distinct Indigenous Australians communities to be 4-fold higher than in Australians of European descent from the same community and region **(Table VI-1)**.[398, 399, 401] Furthermore, the rates of SLE-associated mortality were estimated as 3-fold higher in Indigenous Australians compared to non-Indigenous Australians, although this increased SLE-associated mortality may be confounded by limited access to treatment and services. Nevertheless, these mortality rates mirror the comparative mortality rates of SLE patients of African descent. Compounding evidence, from multiple communities, demonstrates that SLE is more prevalent and potentially more aggressive in Indigenous Australian people, regardless of region, and maps to the observation of other ethnic groups carrying high prevalence of the *CFHR3-1Δ* phenotype.[210, 395-400]

Thus, the higher rates of SLE, combined with lower rates of IgAN and AMD in Indigenous Australians, create a non-infectious disease profile that parallels that seen in non-Australian populations with the *CFHR3-1Δ* haplotype. However, the primary function of complement is to defend against invading pathogens, and the potential impact of a complotype, such as *CFHR3-1Δ*, on infectious diseases in Indigenous Australians needs to be considered.

Table VI-1. Summary of non-infectious complement-associated diseases that disparately affect Indigenous Australians.

The prevalence of systemic lupus erythematosus (SLE), age-related macular degeneration (AMD) and IgA nephropathy (IgAN) in Indigenous and non-Indigenous Australians with expected odds ratios.

Disease	Indigenous Australian Prevalence (%)	Non-Indigenous Australian Prevalence (%)	Odds Ratio For Indigenous vs non-Indigenous	Expected Odds Ratio for *CFHR3-1Δ*$^{-/-}$ †	Age Matched	Location Matched	Reference
SLE	0.05	0.02*	2.5	1.5	No	Yes	[397]
	0.07	0.02	3.5		No	Yes	[396]
	0.09	0.05	1.8		No	Yes	[402]
	0.09	0.02*	4.5		No	No	[403]
Intermediate AMD	5.7	10.5	0.54	0.31	Yes	Yes	[221]
Advanced AMD	0.17	0.96	0.18				
Vision impairing AMD	0.95	10.3	0.0922		Yes	Yes	[404]
IgAN**	19.10	24.40	0.78	0.35-0.56	No	Yes	[391]
RHD	0.6663	0.0109	61.1	NA	Yes	Yes	[405]

AMD = age-related macular degeneration; RHD = rheumatic heart disease

*Estimated national average at time of study

**Frequency in biopsied nephritic patient kidneys

† Determined as $\frac{Disease\ Prevalance(CFHR3{-}1^{-/-})}{Disease\ Prevalance(CFHR3{-}1^{+/+})}$

VI.5 The potential impact of *CFHR3-1Δ* on infectious diseases in Indigenous Australian Communities

The Australian Institute of Health and Welfare report on the overall burden of chronic and infectious diseases among Indigenous Australians highlights alarming infectious disease disparities.[389] In particular, the increased burden of disease associated with two important pathogens, namely *Neisseria meningitidis and Streptococcus pyogenes,* was outlined.[389] Social and environmental factors certainly contribute to the prevalence of these bacterial infections. However, these three organisms are also well described to employ mechanisms for evading complement alternative pathway (AP)-mediated cell killing and thus are advantaged in infecting individuals with a reduced suite of complement proteins.[199]

VI.6 *Neisseria meningitidis*

N. meningitidis is the causal agent of a number of clinically important diseases including meningococcal meningitis, with Indigenous Australians having four times higher rates than non-Indigenous Australians (2.77 vs 0.72 per 100,000 cases),[389, 406] although the fatality rate is comparable in both populations (3-4% of infected individuals).[383] The interactions between *N. meningitidis* and complement are well characterised, where the lectin binding pathway and AP activity are vital for the clearance of this bacteria.[407, 408] To protect itself from complement and subvert the host defences, *N. meningitidis* binds FH, to protect surfaces from complement attack. FH binding to *N. meningitidis* occurs via bacterially expressed FH binding protein (FHbp) (binding FH_{SCR6-7} and $FHR\text{-}3_{SCR1-2}$) and mimicking the sialic acid profile of the host membrane by Neu5Ac, (binding $FH_{SCR19-20}$) to prevent AP-driven complement activation on the bacterial surface. Importantly, FHR-3 normally plays a role in assisting complement-mediated clearance of this pathogen **(Figure VI-2)**.[409] Here, FHR-3 protects the host by competing with FH for binding to FHbp on the surface of the bacteria and preventing FH-mediated protection, consequently leaving the bacteria vulnerable to complement-mediated destruction.[409, 410] Thus, the reduction or loss of FHR-3 functionality as a part of the *CFHR3-1Δ* haplotype is predicted to increase the ability of *N. meningitidis* to bind FH and evade complement AP-mediated killing **(Figure VI-3)**. Therefore, a deficit in FHR-3, as seen in the *CFHR3-1Δ*

haplotype, could subsequently result in greater susceptibility to *N. meningitidis* infection.[409, 411]

VI.7 *Streptococcus pyogenes*

Another infection that heavily burdens the Indigenous Australian community is the Group A Streptococcus, *S. pyogenes.* The complement AP plays a critical role in regulating *S. pyogenes* infections. Similar to *N. meningitidis*, *S. pyogenes* avoids complement-mediated destruction through the bacterial M protein that binds FH and prevents AP activation on the bacterial surface.[412] FH_{SCR6-7} is a crucial region for FH-binding by the *S. pyogene*s M protein. Homologous regions of FH_{SCR6-7} exist as $FHR3_{SCR1-2}$, with >85% sequence homology and similar binding properties.[412] The interaction between the M protein of *S. pyogenes* and FHR-3 has yet to be described but based on this homology to FH, a similar interaction of *S. pyogenes* M protein with FHR-3 is likely, as observed with *N. meningitidis* FHbp (**Figure VI-2**). This prediction is further supported by the observed trends in at least nine other FH-acquiring microbes, such as *Leptospira interrogans, Staphylococcus aureus* and *Plasmodium falciparum* listed in the review by Józsi (2017)[199]. These examples demonstrate that organisms that bind FH, will typically also bind to FHR proteins in homologous regions and that this binding is competitive but does not confer protection from complement. Furthermore, genetically conferred protection from *S. pyogenes* due to a common complement FH haplotype has been described. Haapasalo et al., (2008)[413] demonstrated that the CFH H402 allotype, most notable for increasing risk of AMD, also improves complement-mediated destruction of *S. pyogenes* in patient blood. Interestingly, *CFHR3-1Δ* is in linkage disequilibrium with the FH SNP Y402H, and thus, the *CFHR3-1Δ* alleles occur with Y402 more frequently than the Hardy-Weinberg equilibrium would estimate.[414] It would be expected then that the wild-type (H402/CFHR3-1^{+}) is associated with reduced risk of *S. pyogenes* and increased risk of AMD, but CFH Y402/*CFHR3-1Δ* would be associated with increased risk of *S. pyogenes* infection and reduced AMD. This latter genotype mirrors the phenotype of the studied Indigenous Australian populations – increased risk of *S. pyogenes* infection and reduced risk of AMD. These associations further highlight that the vulnerability of Indigenous Australians to repeated *S. pyogenes* infections may not only be driven by health inequities but also influenced by the *CFHR3-1Δ* haplotype.

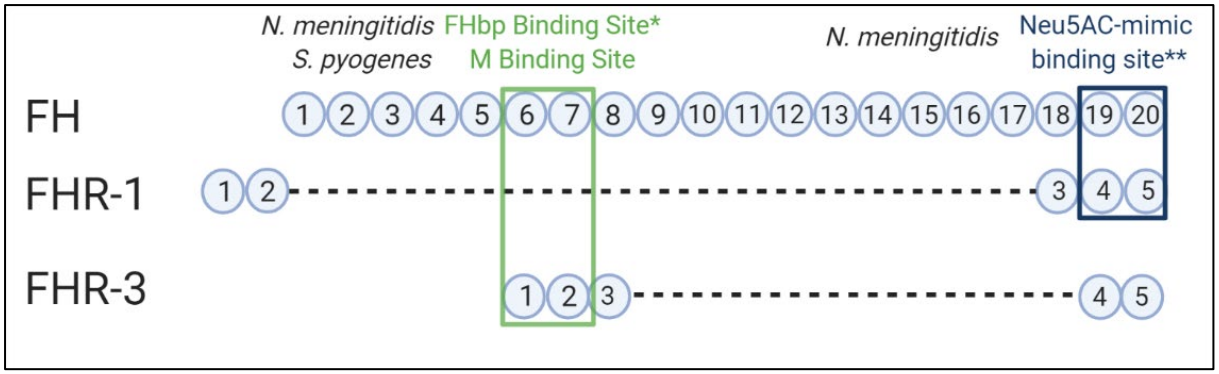

Figure VI- 2. FH has high identity with FH Related proteins 1 and 3 in regions that pathogens bind.

FHR-3 short consensus repeats (SCR) 1 and 2 share high sequence identity with the parent FH SCR6-7 where *N. meningitidis and S. pyogenes* bind by FHbp (confirmed) and potentially M protein (untested), respectively. *N. meningitidis* also binds FH by mimicking human GAG Neu5Ac to bind the sialic acid-binding region of FH (confirmed) and likely the SCR4-5 region of FHR-1 (untested) that shares high identity with the parent molecule FH (>98%). All three bacteria-FH binding sites have been confirmed experimentally. *FHbp-FHR-3 has also been confirmed experimentally **but not Neu5AC-FHR-1 binding for *N. meningitidis*. Only M protein-FH binding has been demonstrated for *S. pyogenes*. FHR = Factor H related, FH = Factor H, FHbp = Factor H binding protein.

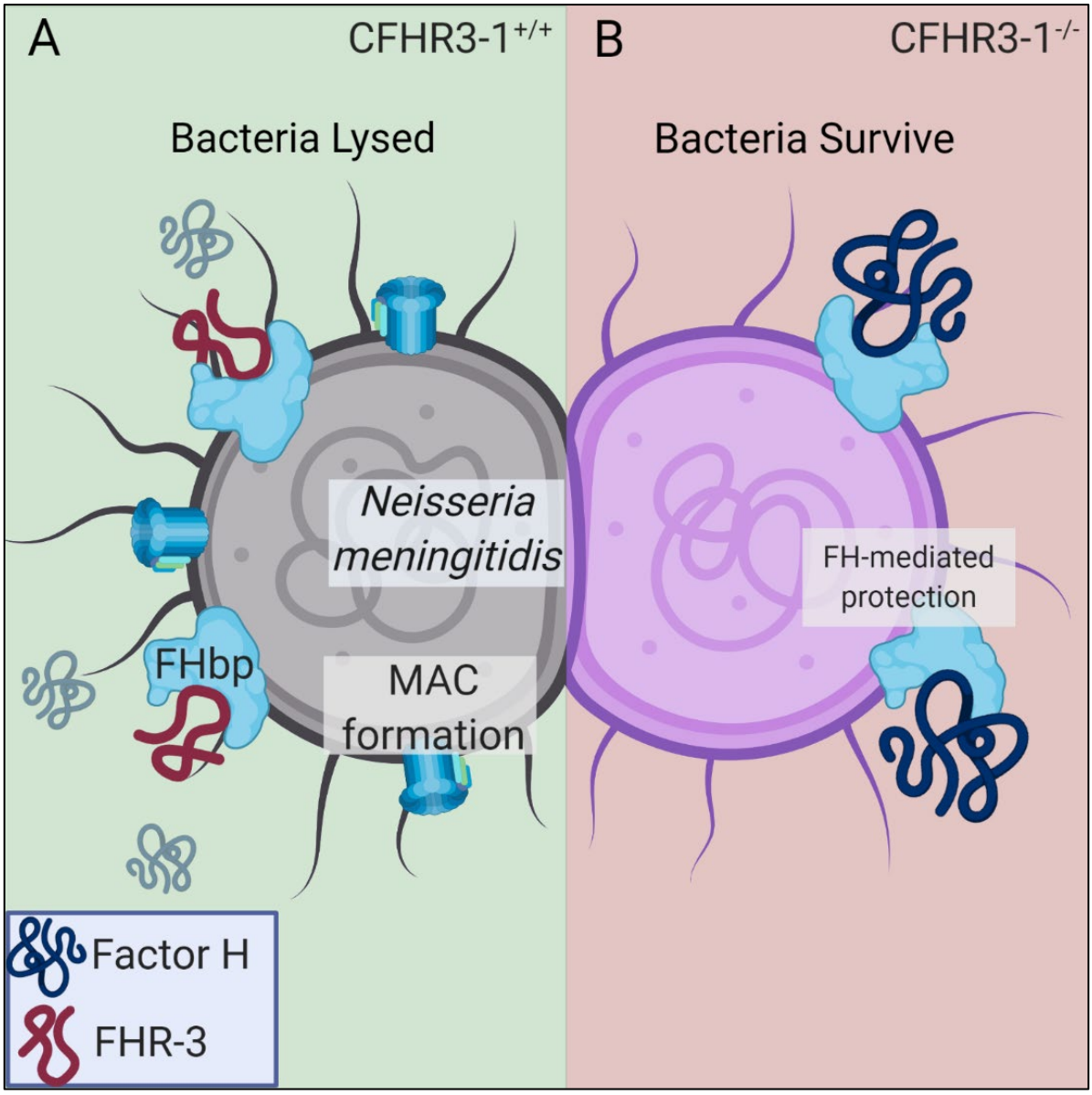

Figure VI-3. FHR-3 competes with FH for the FHbp binding site on *N. meningitidis*.

(A) FHR-3 competes with FH for binding to the bacterial FHbp on the bacterial cell surface, allowing complement to deposit, form the MAC and lyse the bacteria. **(B)** Without FHR-3, *N. meningitidis* binds complement FH via the bacterial FHbp which subsequently protects the bacteria from surface complement activation and cell lysis, leading to bacterial survival. FHR = Factor H related, FHbp = Factor H Binding Protein, MAC = Membrane attack complex.

While *S. pyogenes* infections are problematic, significant health outcomes are impacted by post-infectious sequalae, where antibodies are produced against the *S. pyogenes* M-protein that can cross-react with host tissues.[415]. These immune responses can cause progressive tissue damage, particularly in the heart and kidneys, leading to rheumatic heart disease (RHD) and acute post-streptococcal glomerulonephritis (PSGN), the former dramatically reducing life expectancy among Indigenous Australians.[415, 416]

PSGN causes acute kidney damage that typically resolves within 1-2 weeks. The renal damage is due to antibody-complement complexes forming in the glomerular membrane activating local complement. For PSGN, the disproportionate burden is clear, in a 2018 study 94% of the 323 cases were in Indigenous Australians.[417] Similarly, comparative rates of PSGN in Central Australia revealed a 13.4-fold higher rate (228.7 vs 17 per 100,000) of PSGN in Indigenous Australians compared to non-Indigenous Australians.[416] These data together resemble trends in the Nigerian population, revealing another commonality in disease outcomes, where in Nigeria, PSGN is the leading cause of child morbidity linked to renal disease.[418] Importantly, PSGN is correlated to chronic renal disease later in life, especially when patients have comorbidities such as diabetes and obesity, as is common in the Indigenous Australian population, highlighting the on-going impact of the greater susceptibility to acute *S. pyogenes* infection that may be contributed to through the *CFHR3-1Δ* complotype.[419]

Rheumatic Heart Disease is a second major post-streptococcal sequalae that disproportionately affects Indigenous Australians. In the acute setting, infection by *S. pyogenes* primarily causes a throat infection but in susceptible individuals, generally children and adolescents, infection can manifest as rheumatic fever, characterised by heart valve inflammation, joint inflammation, fever, and rashes.[420] While the majority of symptoms resolve, cardiomyopathy can remain and lead to progressive disability, impacting on the quality of life and premature mortality in young adults. RHD pathology is antibody-mediated, whereby antibodies generated in response to the *S. pyogenes* M-protein are cross-reactive with the cardiac myosin protein in heart valves.[421] If infections are recurrent, the stimulation of immune memory response and increase in cross-reactive antibody production leads to progressive and permanent heart valve damage. Over time, damaged heart valves place individuals at an increased risk of

arrhythmias, stroke, complications during pregnancy and endocarditis.[415, 420, 422, 423] In 2013, a survey of Australians in the Northern Territory revealed that the Indigenous Australian population accounted for 97.6% of RHD cases, despite accounting for only 30% of the surveyed population.[420, 424] In a national registry of newly diagnosed individuals with RHD between 2013 and 2017, 83% (1041 of 1254) were Indigenous Australians.[405, 425] Thus, RHD is clearly a significant and disproportionate problem among Indigenous Australian communities, with rates amongst the highest in the world and comparable to those in Sub-Saharan Africa, with the highest prevalence of at least one *CFHR3-1Δ* allele (50%) worldwide.[422, 423] Although there may not be a direct connection between *CFHR3-1Δ* and RHD, an impact of the *CFHR3-1Δ* complotype in predisposing to repeat *S. pyogenes* infection would indirectly contribute to the development of RHD and thus is critical to investigate.[203]

VI.8 Methods for hypothesis testing

The data presented above on disease susceptibility profiles in Indigenous Australians are highly suggestive of the presence of the *CFHR3-1Δ* genotype, based on known associations in other populations, and mechanisms of the complement system interacting with the pathology of disease. This forms a strong and required justification for directly testing the hypothesis, that Indigenous Australians bear the *CFHR3-1Δ* complotype. A method for evaluating the frequency of copy number variation for *CFHR3-1Δ* is already established and uses a commercial multiplex ligand-dependent probe amplification (MLPA) kit. The premise of MLPA is that gene deletions can be detected through targeted PCR amplification of deletion-adjacent sequences and determining the length between these sites. MLPA was the method used by Holmes et al., (2013) to determine the frequency of *CFHR3-1Δ* in 439 people from 17 nationalities.

The quantitation of *CFHR3-1Δ* in other ethnicities by Holmes et al., (2013)[203] used between 29 and 138 individual samples per ethnicity, depending on availability. Therefore, experiments to test our hypothesis should be performed on at least 30 genomes, preferably in individuals who have at least one parent who identifies as an Indigenous Australian and not from the same family as other participants, to derive an approximate frequency of *CFHR3-1Δ* in members of Indigenous Australian communities. In order to ethically perform genomic research in the Indigenous

Australian community, researcher-community relationship need to be established. Studies and existing databases focused on Indigenous Australian genetic research have paved the way for future research that is collaborative and respectful.[426-429] Retrospective improvements for engagement, involvement, translation, and governance of Indigenous Australian genetic research have also been published.[430] Indigenous Australian complotype research stemming from this hypothesis should foremost benefit and be governed by the communities involved.

VI.11 Summary

Here we have presented observations from clinical data and national health surveillance that support disparate patterns of specific complement-associated diseases and the hypothesis that Indigenous Australians have higher rates of the haplotype *CFHR3-1Δ* than non-Indigenous Australians. Although social and health equity issues are clear contributors to both infectious and non-infectious disease in the Indigenous Australian population, the increased prevalence and morbidity of SLE, *N. meningitidis* and *S. pyogenes* infections, and the decreased prevalence of AMD and IgAN could at least in part be explained by an increased prevalence of *CFHR3-1Δ* in Indigenous Australian communities. This review compiles substantial evidence to justify testing the hypothesis of the presence of the *CFHR3-1Δ* complotype in the Indigenous Australian population and an opportunity to further define the interaction of genotype with the pathology of these important diseases among a vulnerable population. Such knowledge may assist in the development of personalised medicine approaches to target complement factors and improve health outcomes for Indigenous Australians.

CHAPTER VII

GENERAL DISCUSSION AND FUTURE DIRECTIONS

VII.1 DENV remains a global issue

DENV is the most important arbovirus today, as it is endemic in over 100 countries with the potential to infect half of the world's population.[10] Despite attempts as early as 1952 by Dr Albert Sabin, efforts to produce a safe and effective dengue vaccine that can be used without serotyping recipients have been unsuccessful and no approved antivirals have yet been developed.[25, 431-433] Currently, treatment that manages dengue symptoms is the best available practice, although, fluid replacement therapy requires special care, as reperfusion injury can worsen patient outcome.[9] Many countries where dengue is endemic lack the resources to provide healthcare access efficiently and safely to severe dengue patients, especially during outbreaks.[6, 7] Therefore it is clear that further research that attempts to uncover therapeutic targets for dengue is still needed.

VII.2 The complement AP during severe dengue

A potentially life threatening complication of dengue is severe dengue, where the hallmarks of disease are thrombocytopenia and vascular leakage which can cause disseminated coagulopathy, organ failure and hypovolemic shock.[3] Typically, the complement AP fulfills the role of an acute phase immune response by depositing on pathogen surfaces during infection but is heavily regulated on the surface of host-cells to prevent self-directed damage.[147, 148] However, AP dysregulation which may be caused by genetic, autoimmune or infection-associated factors, results in self-directed amplification of the AP which can cause organ damage and potentially death.[289, 350, 414, 434, 435] During severe dengue, circulating FH has been observed to decrease, correlating with decreased AP regulation[217], likely contributing to complement-mediated disease. Furthermore, investigations attempting to define decreased FH in DENV-infected MDM *in vitro* demonstrated that FH mRNA and cell-associated protein increase, but extracellular protein remains unchanged. In consideration of these phenomena it was hypothesised that DENV-infection indeed produces increased FH mRNA and protein, but FH is bound to cell membranes via FH ligands HS and sialic acid. Accordingly, this thesis characterised the stimulation of FH mRNA production, FH transcripts and the translated FH protein, which were defined by various molecular research techniques.

VII.3 FH mRNA and protein increase in response to DENV-infection, induced by secreted stimuli, but FH protein is retained intracellularly

In **Chapter III** of this thesis, publicly available microarray data from dengue and severe dengue patients were utilised to assess changes in transcript abundance for a panel of AP-associated proteins. These investigations revealed that, within cells collected from whole blood, mRNA for FH and FB were indeed increased acutely during dengue. In circulating cells, increased patient FH and FB mRNA were in alignment with previous observations for MDM *in vitro* and the findings in **Chapter IV** using HeLa. As cells in circulation are not typically DENV-infected, potentially changes in mRNA could be in response to secreted stimuli investigated in **Chapter V**.[176, 281-283] In HeLa, it was determined that of the potential FH isoforms, only full length FH mRNA transcripts and protein were produced by DENV-infected cells. In conjunction with FHR mRNA data from **Chapter III** and mass spectrometry data from **Chapter V**, it was reinforced that increased FH mRNA previously observed in MDM was unlikely confounded by FHR1-5 mRNA. Observations of increased FH mRNA and protein in HeLa **(Chapter IV)**, and existing patient data **(Chapter III)**, support the hypothesis that DENV causes induction of both FH mRNA and protein during infection. However, it was determined that increased cell-associated FH was retained intracellularly. Therefore, the original hypothesis that increased cellular FH was cell-membrane bound via ligands such as HS and sialic acid was refuted.

Elevated FB mRNA abundance was observed in the acute phase of dengue patients **(Chapter III).** Additionally, FB protein was uniquely detected by DENV-infected HeLa using mass spectrometry **(Chapter V)** which has also been described previously for MDM and EC *in vitro*[44]. Increased FB availability likely promotes AP activity and amplification during dengue, promoting increased C3 and C5 cleavage products as observed by Nascimento *et al.,* (2009)[217]. However, as increased FH following DENV infection is intracellular **(Chapter IV)**, there is no indication that increased AP activation by FB is met with AP regulation by FH in either infected or bystander cells.

Important contributions were made to the understanding of secreted proteins during DENV infection and their effects on FH mRNA and protein localisation in **Chapter V**. Following DENV infection, it was determined large, secreted, proteins were responsible for FH mRNA induction, but small secreted proteins possessed the ability to increase FH-cell localisation in the absence of virus. Analysis of secreted proteins

released by DENV infected HeLa by mass spectrometry revealed the unique release of complement proteins FB, C5 and vitronectin by DENV infected cells and a large fold-change increase in C4. The number of candidate agents responsible for acute induction of FH mRNA have been narrowed by these analyses and can now be tested systematically. For example, investigating the agents responsible for FH-cell localisation could begin with inhibition of PRRs or cytokine receptor pathways and targeted depletion of cytokines with siRNA or antibodies. Primary candidates for defining FH mRNA production and protein localisation induced by secreted proteins from DENV infected cells include the DENV virion, C5, type I and II IFNs, IL-6 and IL-8, and DENV-NS1.

DENV-NS1 will bind TLR-4 *in vitro*[64], and TLR-4 activation causes downstream translocation of NF-κB to the nucleus.[88] NF-kB activation promotes transcription of cytokines and complement proteins, including FH.[88, 182] Therefore, as FH mRNA was induced by large secreted proteins, further investigation into the relationship between DENV-NS1 are warranted. Thus, the outcomes of this thesis generated a foundation for the hypothesis that DENV-NS1-mediated TLR-4 activation induces FH mRNA production and is the basis of an honours project currently being undertaken by a student. The honours project will focus on the interplay between DENV-NS1, TLR-4 and FH mRNA induction.

VII.4 DENV infection decreases cell membrane GAG abundance, alters FH-membrane binding, and may reduce extracellular FH through induction of CTSL

Using publicly available dengue patient data, transcript abundance for HS-interacting genes and NEU1 were observed to change *in silico* **(Chapter III)** which was corroborated by mass spectrometry in the case of NEU1 **(Chapter V)**. Changes in the abundance of these proteins likely effects the ability of FH proteins to bind cell membranes during infection. Accordingly, DENV decreased cell-associated sulphated GAG abundance, which a significant amount of FH molecules depended on for cell membrane binding during infection but not sialic acid. Importantly, membrane AP regulation by FH is normally dependent on sialic acid binding **(Chapter IV)**.[237] Therefore, a shift in FH binding patterns favouring GAGs and not requiring sialic acid may reveal a mechanism for AP activation on infected cells otherwise protected by FH. AP activation on the surface of mumps virus, measles morbillivirus and respiratory syncytial virus infected cells have all been demonstrated *in vitro*.[436-438] However none

of the publications demonstrating this AP activation addressed the role of FH in these scenarios.[436-438] Perhaps, decreased sialic acid-FH interactions in DENV-infected cells permits AP activation on surfaces without the interference of complement regulation. FH is commonly described to regulate complement on 'self' but not 'altered self' surfaces typically referring to cell death-associated alterations.[160] In this instance, DENV may induce an 'altered self' surface by changing cell surface GAG profiles. Future research that investigates changes in the carbohydrate decoration of DENV-infected cells would provide insight into the recruitment and binding of FH protein and therefore, insight into AP regulation during dengue.

Further, it was observed that dengue patients demonstrated acutely increased mRNA for CTSL, a protein whose importance during dengue, and especially with respect to FH, is of great interest for future investigations. CTSL activation by DENV-NS1 contributes to endothelial glycocalyx degradation[15] and its induction may also provide an explanation for intracellular retention of FH following infection. During apoptosis, FH is recruited and internalised, binding intracellular CTSL.[352] FH directs CTSL-mediated cleavage of C3 to generate iC3b opsonins, promoting phagocytosis of released autoantigens.[352] iC3b can be further converted into C3dg on the surface of healthy cells, which is a much weaker inducer of opsonisation.[439] Therefore, in light of the findings in **Chapter III and IV**, interactions between intracellular CTSL, FH and C3 should be investigated in the context of dengue. Such research should first identify whether intracellular FH-CTSL interactions are indeed occurring during DENV-infection and are directing intracellular iC3b production. If this is true, it would also be important to determine whether iC3b deposition results in viral clearance, promotes phagocytosis of infectious virus, or perhaps protects DENV infected cells from phagocytosis following conversion to C3dg.

VII.5 An proposed model of complement AP during DENV-infection

In consideration of the results herein, combined with the current understanding of dengue and complement AP regulation these observations allow for the development of an updated hypothetical model of complement-mediated dengue pathology **(Figure VII-1)**. This model highlights the interactions between the DENV virion and DENV-NS1 with PRRs and the cascade of gene transcription during infection. Following induction of complement genes and cytokines, proinflammatory cytokines and complement AP

activating proteins are increased, the latter initiating the AP. The AP is largely uninhibited due to intracellular retention of FH until the MAC formation stage, which is blocked by DENV-NS1 and the host protein vitronectin. Failure to regulate C3- and C5-convertase formation and activity results in anaphylatoxin production. Together, DENV-NS1 and host vasoactive molecules produced in response to infection are proposed to induce endothelial hyperpermeability.

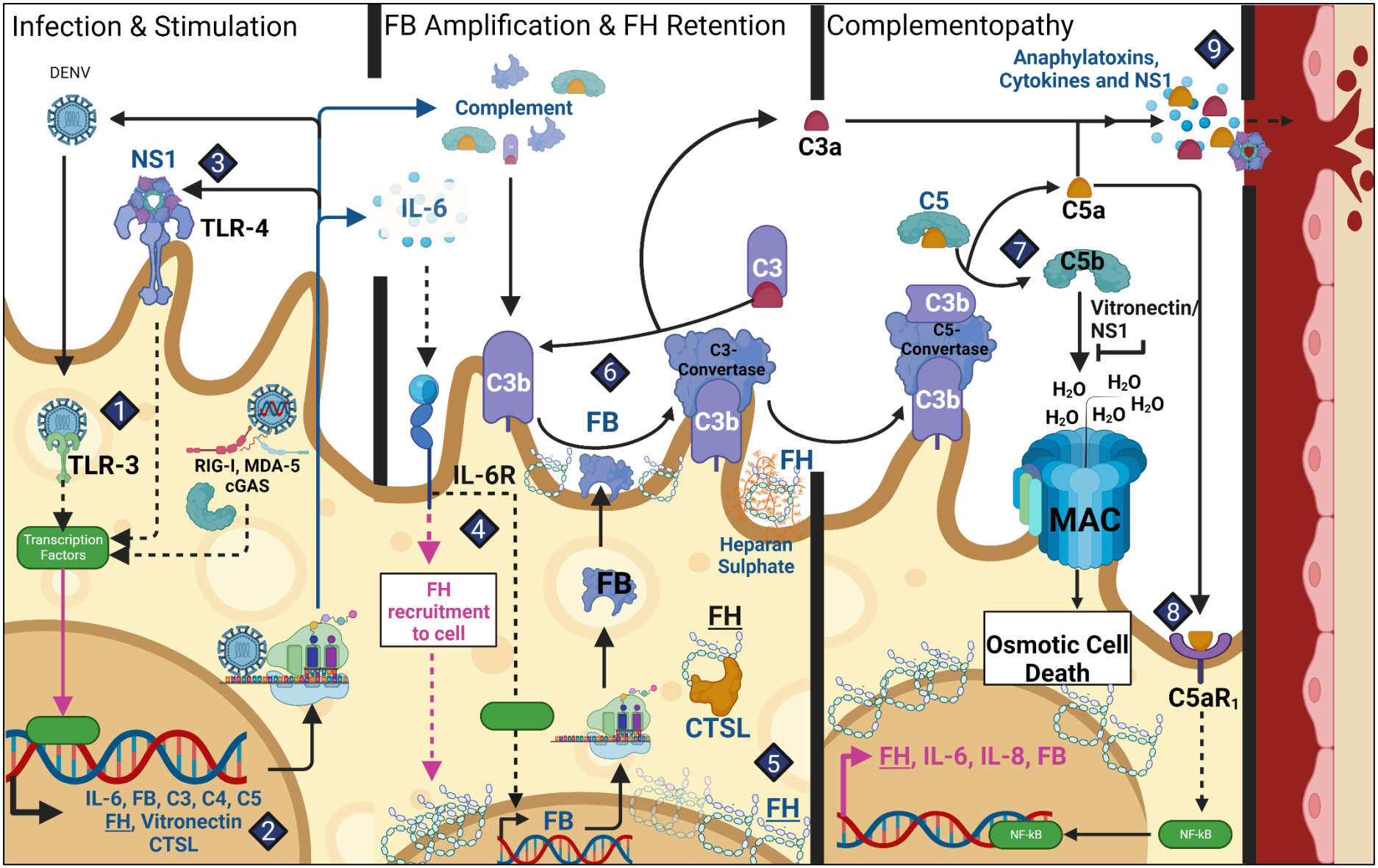
Infection & Stimulation
FB Amplification & FH Retention
Complementopathy
DENV
NS1
3
TLR-4
Complement
IL-6
C3a
Anaphylatoxins, Cytokines and NS1
9
C5
C5a
7
C5b
C3
C3b
C3- Convertase
C3b
6
FB
C5- Convertase
C3b
Vitronectin/ NS1
H_2O H_2O H_2O H_2O H_2O
1
TLR-3
RIG-I, MDA-5 cGAS
IL-6R
FH
Heparan Sulphate
MAC
Transcription Factors
4
FH recruitment to cell
FB
FH
CTSL
Osmotic Cell Death
8
$C5aR_1$
5
FH, IL-6, IL-8, FB
IL-6, FB, C3, C4, C5
FH, Vitronectin
CTSL
2
FB
FH
NF-kB
NF-kB

Figure VII-1. A proposed model of dengue-mediated activation but not regulation of the complement alternative pathway and its effects on vascular permeability.

This model proposes: **(1)** DENV infection stimulates dsRNA and dsDNA intracellular PAMP receptors, RIG-I, cGAS and MDA-5 that **(2)** activate downstream transcription factors to induce IL-6 **,** IL-8, FB, C3, C4, C5, FH, vitronectin and CTSL. **(3)** Newly translated and secreted NS1 will activate TLR-4 and also cause downstream transcription factor activation, resulting in the transcription of FH. **(4)** IL-6 activates IL-6R, inducing FB transcription, translation, and FH recruitment, **(5)** where FH is confined to the intracellular space, potentially by CTSL. **(6)** Complement proteins in circulation and produced following infection, promote C3-convertase (C3bBb) production on host cells, amplifying feedback of C3 cleavage into C3b, producing more C3-convertases and releasing C3a in the process. **(7)** Complement C5 is cleaved into C5b by the C5 convertase (C3bBbC3b) and releases C5a. C5b initiates formation of the MAC, which normally fenestrates the membrane and causes osmotic cell death, but in the context of DENV infection MAC polymerisation is inhibited by NS1 and increased vitronectin. **(8)** C5a can be sensed by the $C5aR_1$ on the outside of cells which causes downstream activation of NF-κB and induces further FH mRNA transcription. **(9)** NS1, C3a and C5a, and cytokines such as IL-6 and IL-8 are potent inducers of vascular permeability and in high concentrations will induce vascular hyperpermeability. Solid lines represent movement and binding. Dashed lines represent a molecule eliciting an effect. Black text is based on existing literature only, dark blue text represents the novel findings of this thesis and validations of existing literature by this project. Pink text represents hypothetical additions to the model based on the observations herein. DENV = Dengue Virus, cGAS = Cyclic GMP-AMP Synthase, RIG-I = Retinoic Acid Inducible Gene – I, MDA5 = Melanocyte Differentiation-Associated Protein 5, TLR = Toll-like Receptor, FH = factor H, FB = Factor B, CTSL = Cathepsin L, IL = Interleukin, IL-6R = Interleukin-6 Receptor, NS1 = Non-structural protein 1, NF-κB = Nuclear Factor kappa-light-chain-enhancer of activated B cells, MAC = Membrane attack complex, $C5aR_1$ = C5a Receptor 1.

VII.6 The future of dengue therapeutics targeting the complement AP

Aberrant AP activation causes diseases that severely reduce quality of life and life span, such as atypical haemolytic uraemic syndrome (aHUS) and paroxysmal nocturnal haemoglobinuria (PNH).[289, 440] Today, therapies to treat each of these diseases have been approved but are too expensive to trial in dengue patients due to the inherent costs of production and the orphan status of aHUS and PNH.[441] Eculizumab is a monoclonal antibody to treat aHUS and PNH approved in 2011 that binds C5, preventing C5a generation and membrane attack complex formation.[442] A decade later, the pegylated pentadecapeptide Empaveli (Alexion) was approved by the FDA to treat PNH.[443] Excitingly, this ablates complement activation by targeting C3 and is not a monoclonal antibody.[443] Other therapeutics in development are aimed at treating diseases such as C3 glomerulonephropathy and AMD through exogenous recombinant FH proteins.[444-446] Thus, with these two approved drugs and many others undergoing clinical trial such as avacopan, zilucoplan and conversin to treat PNH, the field of therapeutics to treat complementopathy is expanding.[447-450] Potentially, Eculizumab, Empaveli or related biologics may be trialled to treat dengue in the future if the issues of cost can be overcome. As FH-cell binding has been observed for MDM and HeLa following FH mRNA induction, the results of this thesis highlight that full length FH protein is increased but retained intracellularly and promoting its release may be key to resolving dengue induced complementopathy. Alternatively, there is no known disease associated with an overabundance of circulating FH, reinforced by the observation that FH can vary 5-fold between adults.[184] Therefore, provision of exogenous FH or smaller biologics that contain FI cofactor domains, but not host-cell binding domains may also remedy the complement-mediated disease observed in dengue patients. Of course, exogenous FH may suffer the same pitfalls as endogenous FH, and be rapidly recruited to cells rather than acting in circulation. However, there is no clearly apparent risk in trialling provision of exogenous FH in severe dengue patients and is therefore worth considering further. No evidence suggests DENV virions escape virolysis via FH binding as described for other pathogens, nor does FH totally protect infected cells in other viral infections.[436, 438] Therefore, it is unlikely that increased host-specific complement regulation performed by increased FH will prevent complement activity from killing infected cells. Shifting the focus from FH, it would be interesting to determine the importance of FB in protection from DENV. This could be performed *in vitro,* where the novel small molecule FB inhibitor, iptacopan could be used and replication of DENV defined. Here, if it can be

demonstrated that FB is not critical in defending against DENV, reducing FB rather than increasing FH may prove a viable option to target AP dysregulation and disease.

VII.7 The evidence correlating Indigenous Australian complotypes with complement-mediated diseases

Due to pandemic related constraints, the ability to perform wet-lab experimental research between 2020 and 2022 was impaired. Serendipitously, in communication with Professor Jamie Craig and Mr Jose Estevez, it came to my attention that trends in disease outcomes for Indigenous Australians resembled trends of other people groups which was correlated to copy number variants of *CFHR* genes. However, no literature that characterised the Indigenous Australian complotype or AP regulation could be found. The complotype can vary greatly between ethnicities[203] and Indigenous Australians had been isolated from other populations for approximately 60,000-75,000 years.[451] Thus, it was clear the diversity of the Indigenous Australian complotype needed to be investigated further. It is unethical to perform medical research in Indigenous Australian communities without first acquiring their approval. Thus, a literature review was undertaken to define the diseases that interact with complement and disparately affect Indigenous Australians. Although the conclusions drawn from this investigation focus on deletion of *CFHR1* and *CFHR3*, understanding drawn from researching complement in infectious diseases and complementopathies demonstrate that the complotype of a people group is highly variable.[182, 203, 243] Therefore, the true cause for the phenomena of high SLE but low AMD and IgAN in Indigenous Australians may be the result of variants in other complement genes. However, the compilation of available data surrounding AMD, IgAN, SLE and infectious disease sequalae such as RHD and PSGN are compelling. Therefore, these data at least lay precedence for investigating the Indigenous Australian complotype further. With the support of the literature review in **Chapter VI**, future research, performed respectfully, and with the approval of the Indigenous Australian community, could define copy number variation of *CFHR3-1Δ* in Indigenous Australians. Following this, definition of the RCA on chromosome 1 through GWAS and quantitation of serum FH by ELISA could elucidate the complotype of Indigenous Australians further. These findings could then be paired with the rates of disease prevalence that disproportionately affect people of Indigenous Australian descent to inform clinical decisions. Future projects could use these data to evaluate the benefit of therapeutics to replace the role of FHR-1 and FHR-3 during infection or investigate bactericidal activity against clinically relevant pathogens. As a result, investigations grounded in the findings of this literature review and

proposed future research will better inform risk management and therapeutic care of Indigenous Australians. The benefits of understanding Indigenous Australian complotypes will extend to the diseases listed herein, as well as many other complementopathies and infectious diseases.

VII.8 Conclusion

This project has investigated the role of FH and related proteins during DENV-infection and brought together literature surrounding Indigenous health, FHRs and complementopathies more broadly. Molecular research revealed that FH mRNA, which is inducible by large secreted proteins produced during DENV-infection results in FH protein that is retained intracellularly *in vitro*, potentially contributing to decreased FH in patients. These outcomes suggest that increased mRNA for FH will not increase regulation of local extracellular AP activity. This study also defined that HeLa mimicked the patterns of FH mRNA and protein production of MDM during DENV infection and displayed induction of secreted proteins consistent with observations in dengue and severe dengue patients. Therefore, HeLa will be a valuable tool for future characterisation of complement and potentially the secreted proteins produced in response to DENV. Altogether, this thesis has demonstrated DENV induces FH mRNA production without secreted protein and that future therapies to treat the complement-mediated symptoms of dengue may succeed through promoting release of intracellular FH. The specific nature of AP regulation by FH on healthy cells, with no evidence that DENV evades complement via FH, suggests increased FH would not compromise complement-mediated immunity. Increasing FH-mediated regulation early in infection is therefore likely to reduce the burden of complement activation on the vascular endothelium, potentially reducing the risk of severe dengue. As DENV remains a global burden, targeting mechanisms of AP mediate pathology by increasing complement regulation is an exciting avenue for future therapeutic design.

APPENDIX

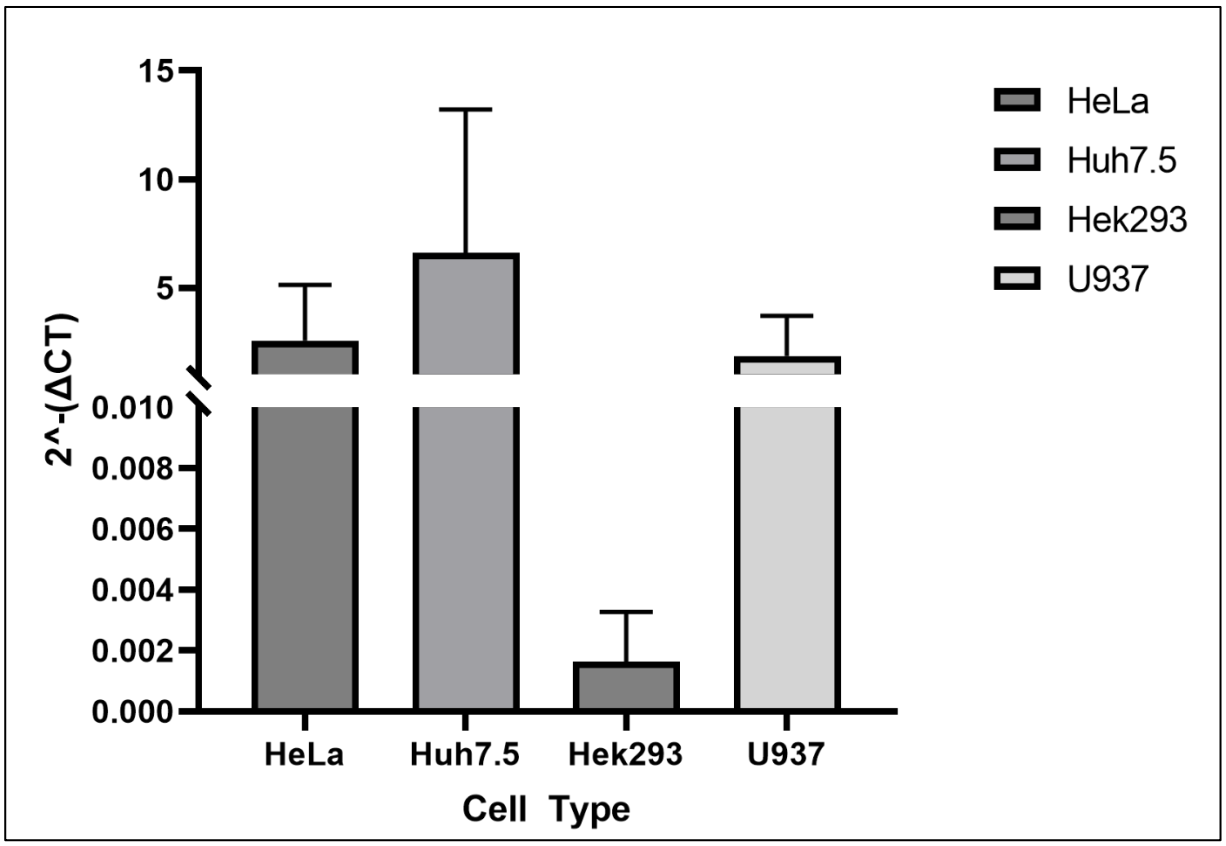

Figure A. 1 HeLa, Huh7.5 and U937 produce highly equivocal DENV RNA by 48 hpi. Cells were infected with DENV-2 (MOI=1) in separate experiments and incubated for 48 hours. Cells had RNA harvested and DENV RNA was detected by RT-qPCR. Results represent the 2^(-ΔCT) of DENV RNA normalised to the housekeeping gene cyclophilin. Results are not statistically comparable across cell lines as separate experiments and are reported together for qualitative comparison only.

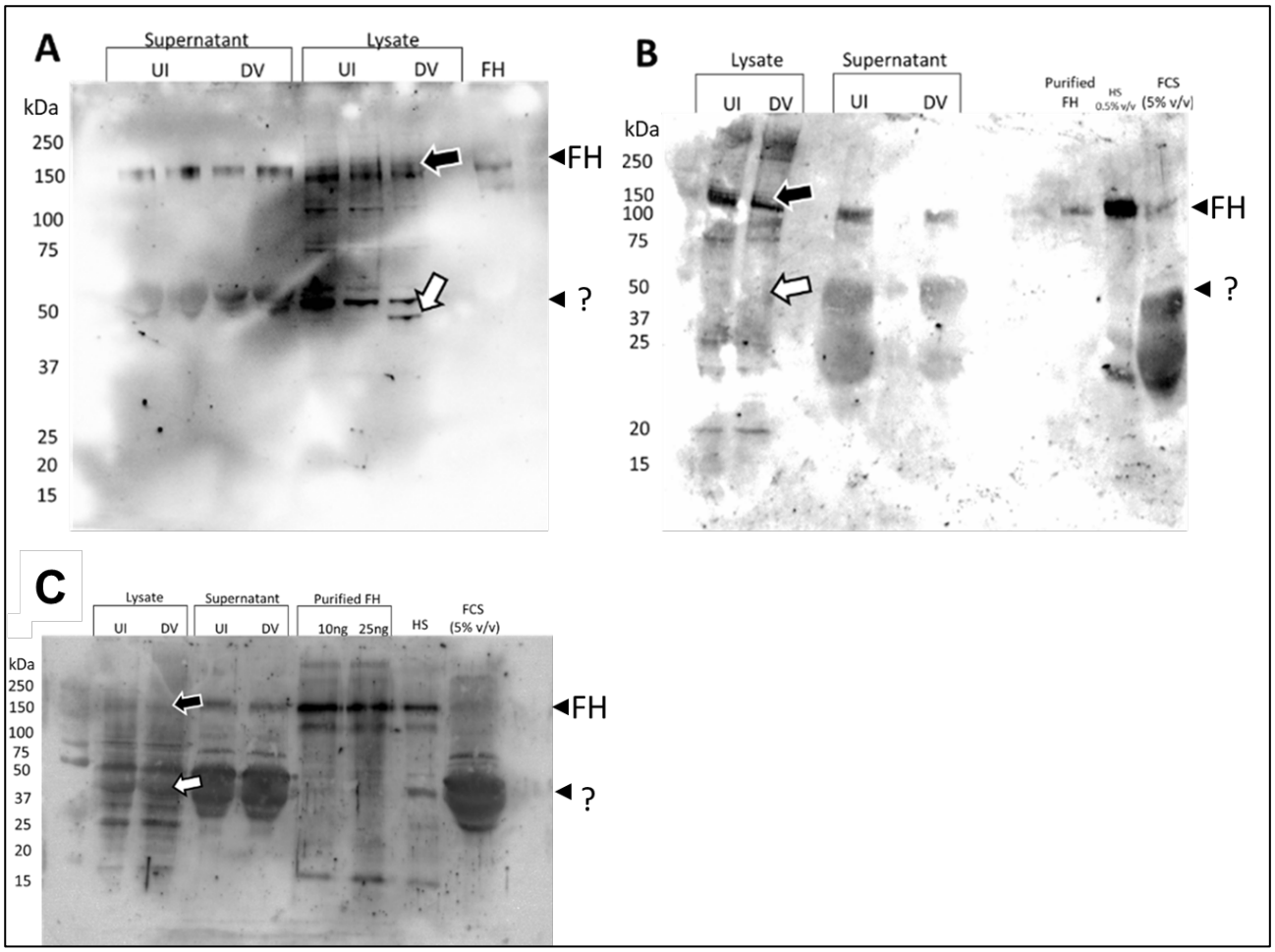

Figure A.2. Western blot does cannot facilitate discernment of 10 ng and 25 ng of FH and FH-reactive protein species at 50 kDa are not consistently detectable.

(A-C) The 50-55 kDa molecular weight bands (White arrows) were not reliably observed (3/5 experiments), even when identical samples were used for repeat Western blots. Black arrows represent the expected location of full length FH (150 kDa) (C) Purified FH was not discernibly different when analysed by Western blot at 10 and 25 ng of loaded protein (equivalent to the mean FH quantitated by ELISA).

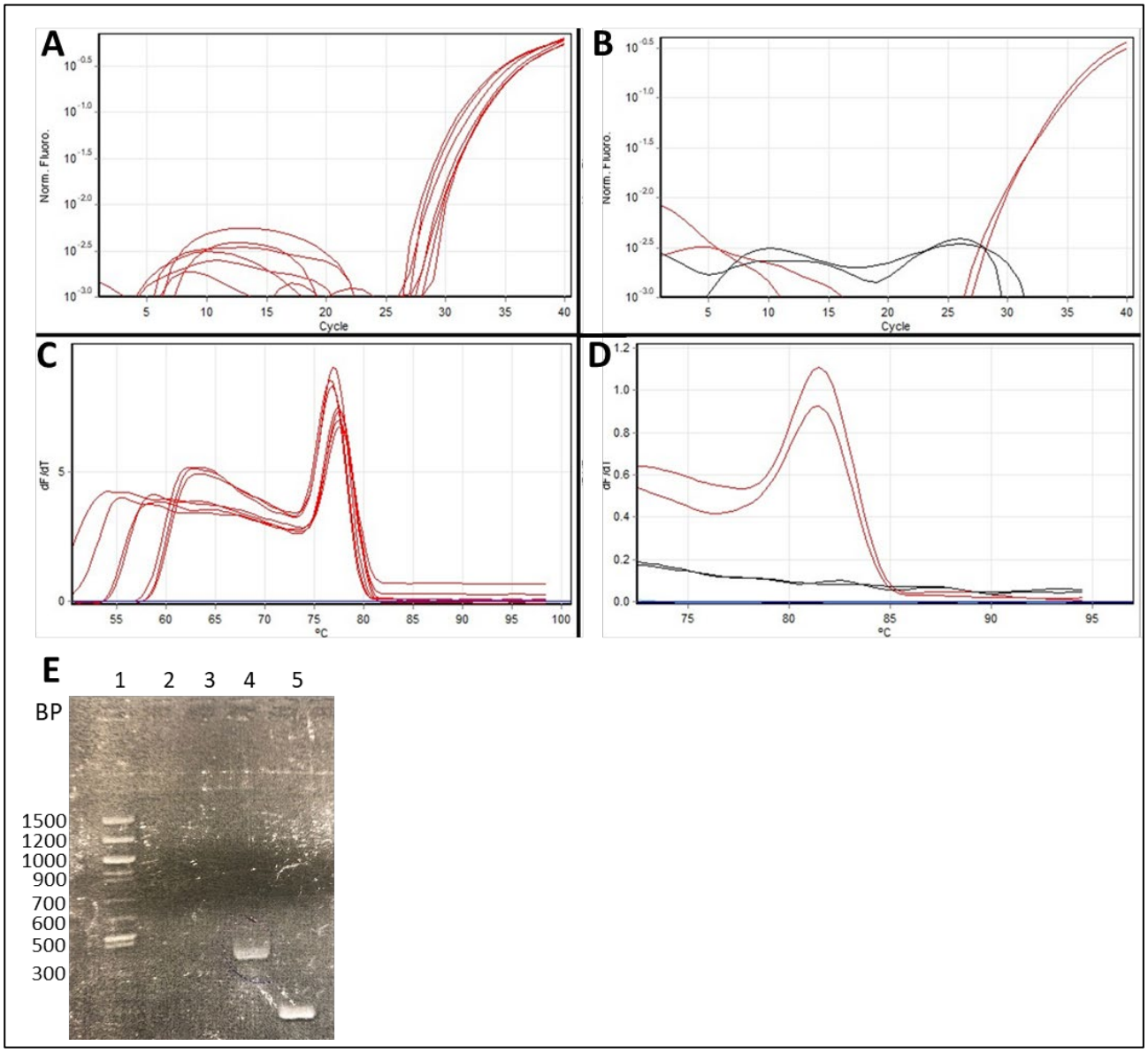

Figure A. 3. FHL-1 and FHR-1 primer validation in Huh7.5.

Huh7.5 were lysed in TRIzol and had RNA extracted which was subject to RT-PCR. cDNA was then analysed by qPCR using **(A+C)** FHL-1 and **(B+D)** FHR-1 specific primers **(Table II-3)**. **(A-D)** Red lines represent Huh7.5 samples, black lines represent no template controls. **(E)** Amplicon size was estimated by agarose gel electrophoresis. Lane 1: NEB 100bp ladder, Lane 2: blank well, Lane 3: No template control, Lane 4: FHR-1 amplicon, Lane 5: FHL-1 amplicon. **E** is a photograph of an image, as the digital file was corrupted.

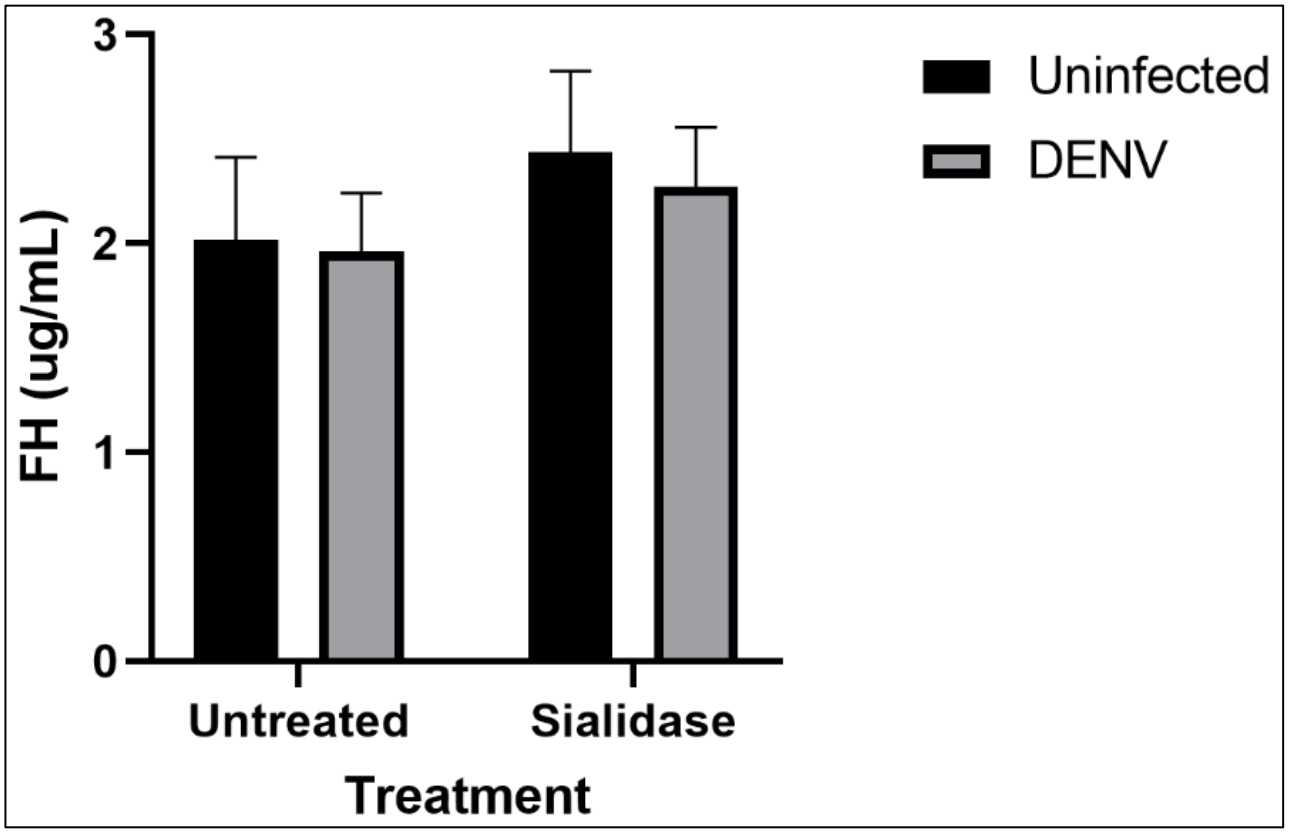

Figure A.4. FH binding is not dependent on cell surface sialic acid alone.

HeLa were either uninfected or infected with DENV (MOI=1) for 48 hours. Following infection, cells were treated with neuraminidase (*C. perfingens)* 90mIU/mL for 1 hour and free FH quantitated by ELISA. FH = factor H, DENV = Dengue Virus.

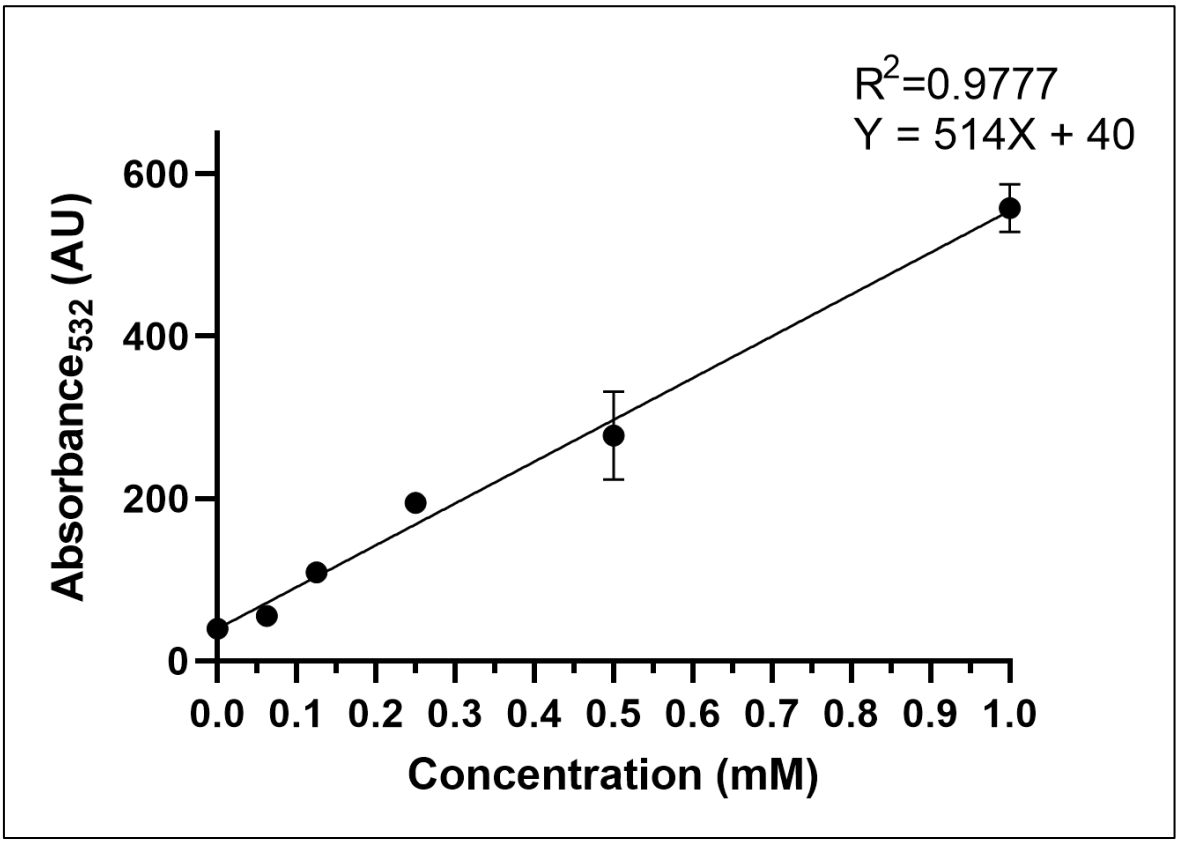

Figure A.5. Validation of the thiobarbituric acid assay for malodnialdehyde quantiation.

Thiobarbituric acid assay was performed on known concentrations of malondialdehyde and changes in absorbance at 532 nm used to determine the line of best fit. Assay was performed with three technical replicates per concentration and represents one of three assays with the same outcome.

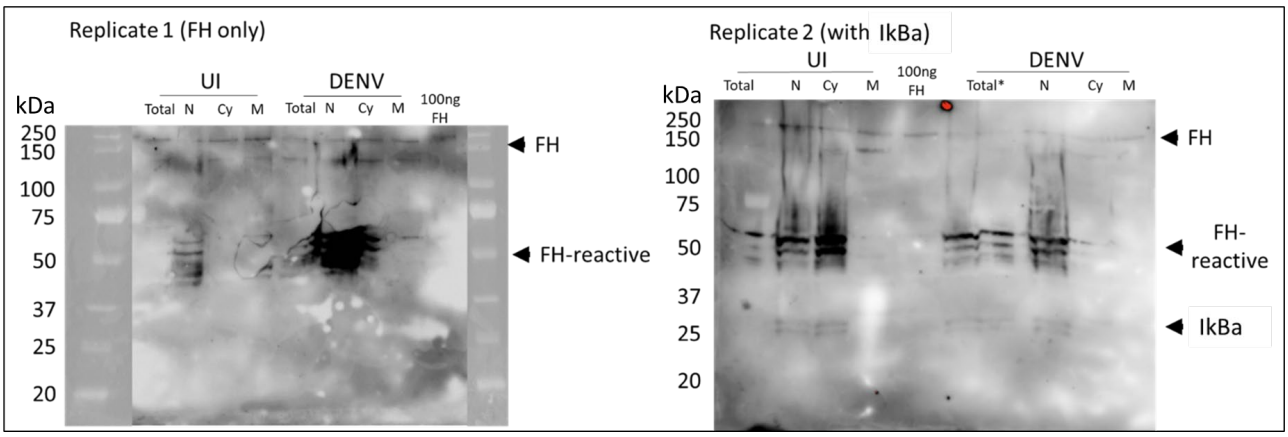

Figure A. 6. Cytosolic protein IkBα contamination is detectable in the nuclear fraction of fractionally centrifugated cell lysates.

HeLa were either uninfected or infected with DENV MOI=1 for 48 hours. Cells were lysed and lysates underwent fractional centrifugation to generate nuclear, cytosolic and membrane fractions which were compared by Western blot and membrane probed for FH and IkBa. Results represent one experiment with FH probing only (left) and an independent second experiment probed for both FH and IkBa (right). N = nuclear, Cy = cytoplasmic, M = membrane, FH = Factor H, DENV = Dengue virus.

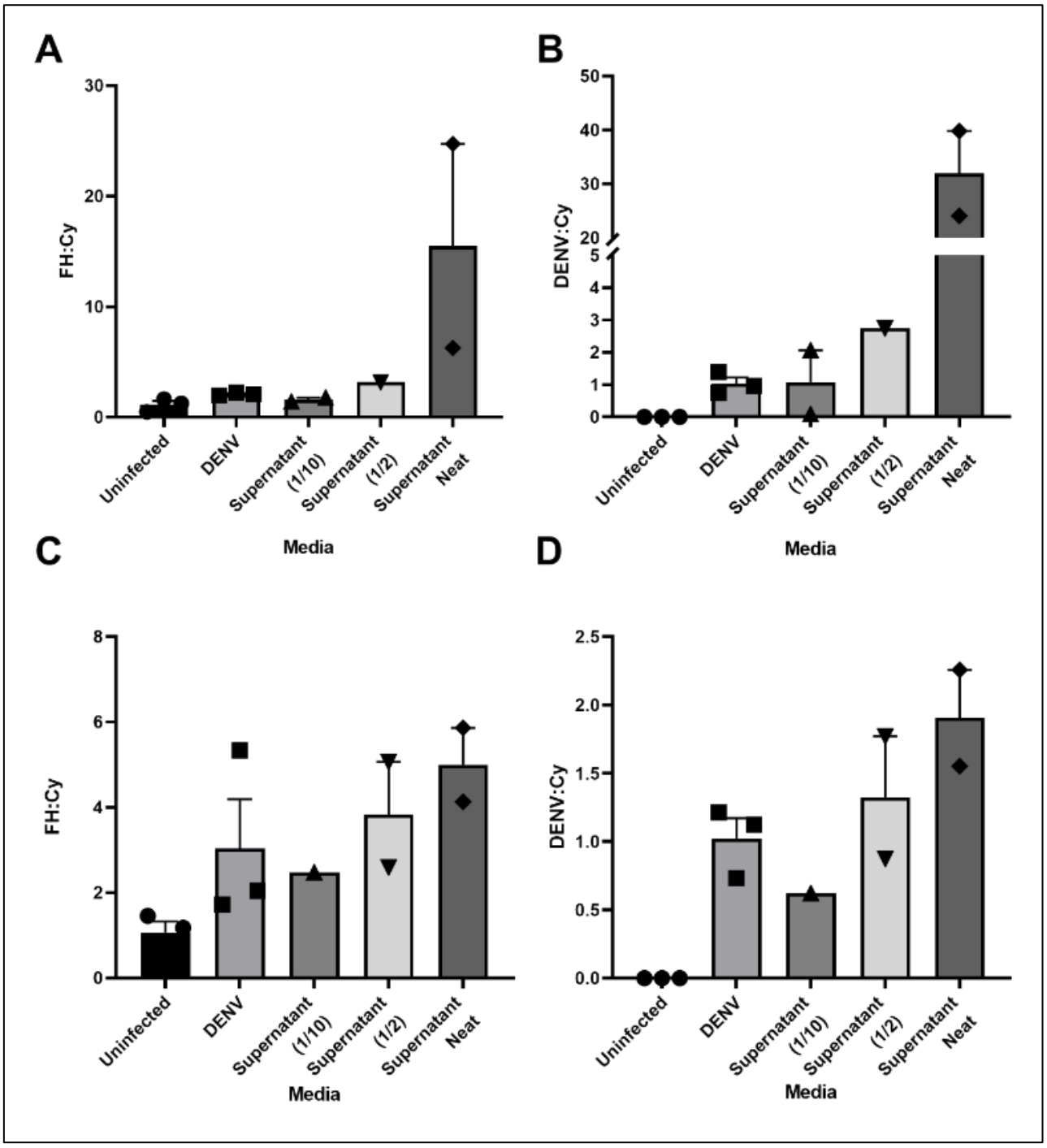

Figure A. 7. Supernatant of DENV-infected HeLa induce a trend for increased FH in a dose dependent manner by 24 hpi.

HeLa were treated with conditioned media, infected with DENV (MOI=1) or supernatant from DENV-infected HeLa. RNA was harvested at **(A+B)** 24 and **(C+D)** 48 hours following treatment and was analysed by RT-qPCR for **(A+C)** FH or **(B+D)** DENV. Values were normalised to cyclophilin and are expressed relative to uninfected controls. Results are representative of one experiment, performed with n=3 biological replicates for UI and DV, n=2 for each supernatant treatment. One-way ANOVA with Bonferroni corrections. FH = Factor H, DENV = Dengue Virus, Cy = Cyclophilin.

Table A.1 Price and time investment to quantitate sialic acid comparing existing assays. Prices taken directly from supplier websites. Time to perform experiments as per manufacturer's instructions. Highlighted cell (top) is the novel sialic acid binding assay developed in **Chapter IV**.

Product	Supplier	Cost/sample (AUD)	Incubation Time to Perform Assay (h)	Intended/advertised sample type	Validated for cell membranes/lysates?	Available in Australia?
Novel sialic acid binding assay	N/A	$0.56	50 mins	Fixed cells	Yes	NA
Sialic Acid Assay Kit LS-K187	LSbio	$10.01	120 mins for bound sialic acid or 60 mins for free sialic acid	Bound or Free sialic acid	Yes	No
Sialic Acid Quantitation Kit KE-SIALIQ	QA bio	$33.84	4 hours ('Method 3', Glycoprotein digestion) or overnight ('Method 4', Whole cells)	Glycoproteins or whole cells	Yes	Yes
Sialic Acid Assay Kit MAK314-1KT	Merck	$11.28	80 mins	Biological fluids	Yes	Yes
QuantiChrom™ Sialic Acid Assay Kit - DSLA-100	BioAssay Systems	$6.61	80 mins	Biological fluids	Yes	Yes
Sialic Acid (NANA) Assay Kit (ab83375)	abcam	$8.48	40 mins	Culture supernatant and Urine	No	Yes

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